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American Journal of Veterinary RESEARCH

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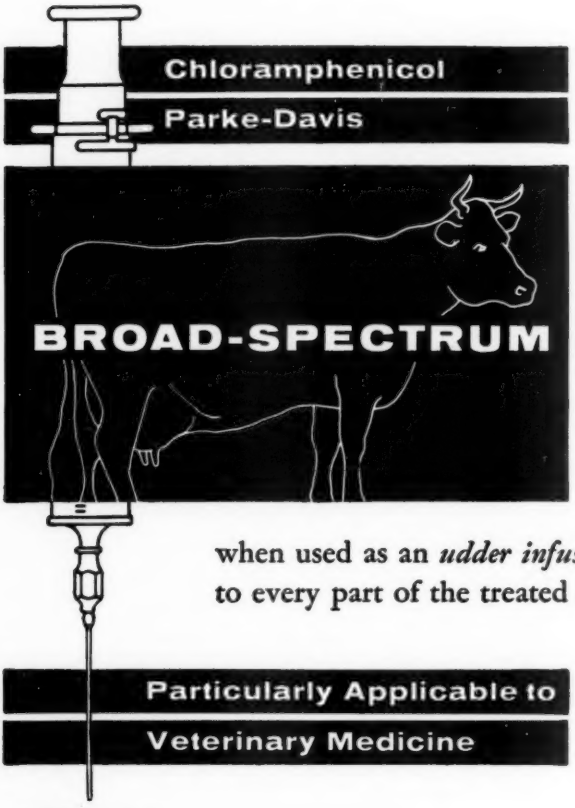

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Volume XXI January, 1960 Number 80

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The recipient of a fellowship must be a veterinarian and a citizen of the United States or Canada. Veterinary students who expect to graduate at the end of the current school year and who wish to follow a career in research may apply for a fellowship.

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The Committee on Fellowships of the Council on Research will meet in March to consider applications, and the awards will be announced soon afterward. The stipend will be determined in each case by the needs of the individual, the location of the school in which he proposes to work, and other factors. In general, the stipends range from $100 monthly and upward.

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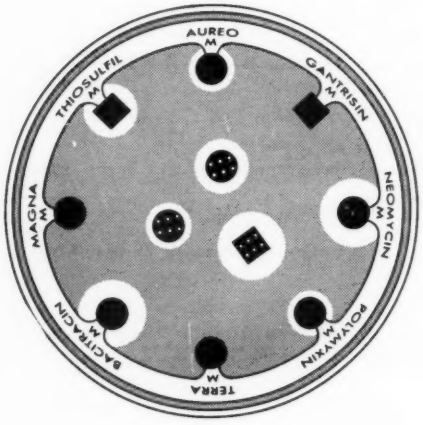

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Postnatal Tooth Development in Cattle

W. A. BARRY BROWN, L.D.S.; PAUL V. CHRISTOFFERSON, D.V.M.;
MAURY MASSLER, D.D.S., M.S.; MARVIN B. WEISS, D.D.S.

Chicago, Illinois

INTEREST in the teeth of cattle dates from the latter half of the nineteenth century when disputes as to the age of veal carcasses were settled by the state of eruption and amount of wear of the incisor teeth.[5] Recently, additional attention has been drawn to the teeth of cattle by the recognition of the role played by fluorides in the mottling of the teeth. Many studies have been done to classify and analyze the effect which dietary fluorides have on the teeth.[4] It is also recognized that other systemic factors may influence the quality of the dental structures. However, these studies are hampered by a paucity of knowledge concerning the details of tooth development in cattle, and especially the exact chronology of enamel development. Without an exact knowledge of the chronology of tooth development, it is not possible to make an accurate correlation between the systemic ailment and its effects on the teeth. Except for Garlick's study in 1954,[3] few controlled studies have been conducted relative to the development of teeth of cattle.

PURPOSE

The primary purpose of this study was to examine and describe the development, erupton, and resorption of the teeth in cattle, with special emphasis on the *chronology of enamel and dentin development in the mandibular incisor and canine teeth.* It was hoped thereby to provide a baseline of reference for future studies in tooth ring analysis, *i.e.*, effects of various systemic factors such as fluorosis, nutritional deficiencies, and infectious diseases upon tooth development in cattle.

A secondary, but important, purpose of this study was to determine whether tooth development was strongly influenced by genetic differences. Several breeds of dairy and beef cattle were examined radiographically to determine whether the different breeds have significantly different chronological schedules. If they had, this might be due to genetic difference. If, however, the schedules were similar, it must be concluded that tooth development probably follows a fixed schedule and is not likely to vary in different genetic strains.

This study presents the chronology of crown and root formation for the four permanent teeth in the anterior portion of the mandibles of 869 purebred dairy and beef cattle. The chronology of lower premolar and molar development is also presented. The chronology of tooth development was found to be similar in the four dairy and three beef breeds of cattle studied. None showed any consistent precociousness or retardation of any one of the developmental processes. Interestingly, no consistent differences were found in the chronology of tooth development between the bulls and the cows.

Part of a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science in Pedodontics, University of Illinois, Chicago, Ill. (present address, the London Hospital Dental School, Whitechapel, London E. 1, England). Dr. Christofferson is veterinarian for the Agricultural Division, Geneva Works, Columbia-Geneva Division of U.S. Steel Corporation, Provo, Utah; Dr. Massler, professor and head and Dr. Weiss is research associate, of the Department of Pedodontics, University of Illinois College of Dentistry, Chicago, Ill.

This study was supported in part by a generous grant from the Columbia-Geneva Division of U.S. Steel Corporation.

A Review of Tooth Development

ENAMEL AND DENTIN FORMATION

The fundamental stages of anterior tooth development in cattle are essentially the same as have been described in human teeth.[3] The life cycle of a human deciduous incisor is shown (fig. 1). Schour and Massler have divided tooth development into the following stages:

1) *Growth*
   - *a*) *Initiation* of cells from the oral epithelium to form tooth buds.
   - *b*) *Proliferation* of tooth bud cells to form the cap stage of development.
   - *c*) *Histodifferentiation* of cells into pre-enamel and pre-dentin forming cells.
   - *d*) *Morphodifferentiation* and alignment of cells to outline the size and shape of the future tooth.
   - *e*) *Apposition* of enamel and dentin matrix by ameloblasts and dentinoblasts.
2) *Calcification* of the enamel and dentin matrix.
3) *Eruption** of the tooth toward and into the oral cavity.
4) *Attrition* or abrasive wear of the teeth.
5) *Resorption* of the deciduous root and *exfoliation* of the tooth.
6) Repetition of the above cycle in each permanent tooth (except resorption and exfoliation).

The enamel which forms the outer cap of the crown is derived from ectodermal cells which line the oral cavity. The underlying mesoderm differentiates into the dental pulp which, in turn, forms the central core of dentin.

a) *Initiation.*—The life cycle of a tooth begins *in utero* with a thickening of the oral epithelium. The cells multiply and form a tooth bud (A of fig. 1).

b) *Proliferation.*—Unequal growth of the tooth bud leads to the cap stage (B in fig. 1). Changes then take place in the inner and outer layers of epithelium and in the cells between. When these occur, this epithelial structure is known as the "enamel organ" and is composed of an outer and inner enamel epithelium with an enclosed stellate reticulum. At the same time, the cells of the mesenchyme which are enclosed by the epithelial cap condense to form the dentin-forming organ or "dental papilla." The mesodermal cells around and outside the enamel organ condense to form the dental sac which will contribute cells for the development of the periodontal membrane.

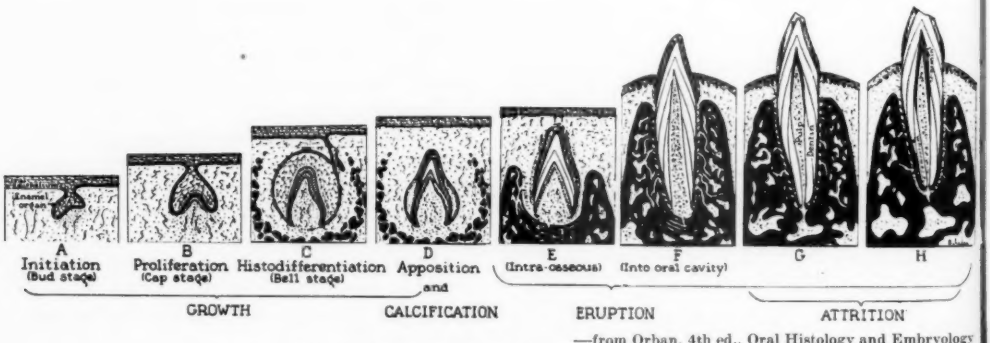
c) *Histodifferentiation.*—The cells of the enamel- and dentin-forming organs undergo marked histologic changes and become specialized to form the enamel and dentin matrix (the bell stage of the tooth germ, C in fig. 1). The layer of inner epithelial cells of the enamel organ differentiate just prior to amelogenesis into tall enamel-forming cells, the ameloblasts. As soon as this occurs, the subjacent mesenchymal cells of the dental papilla differentiate into odontoblasts, the dentin-producing cells. The stratum intermedium is a layer of cells that forms from the stellate reticulum next to the inner enamel epithelium. They apparently are part of the enamel-forming mechanism.

When the crown of the tooth is completed, the inner and outer enamel epithelium proliferate at the margins of the bell-shaped enamel organ and give rise to Hertwig's epithelial sheath. This grows downward to outline the dentino-cemental junction and the future root (E in fig. 1).

d) *Morphodifferentiation.*—As the formative cells differentiate, they align themselves along the future dentino-enamel and dentino-cemental junction and thus establish the size and shape of the future crown and root just before beginning the deposition of enamel and dentin matrix. Thus *the final size and shape of the tooth are already determined before deposition of enamel and dentin commences.*

e) *Apposition.*—This is a regular and rhythmic deposition of the extracellular material by the enamel- and dentin-forming cells.

* Eruption refers to the physiologic tooth movements through the jaw bone and after emergence into the oral cavity.



—from Orban, 4th ed., Oral Histology and Embryology

Fig. 1—Life cycle of the human incisor tooth.

Fig. 2—Mixed anterior dentition (teeth occluding with maxillary pad.)

Amelogenesis is a two-phase system consisting of a deposition of the enamel matrix followed by a maturation or calcification of this organic matrix. The enamel matrix is formed of enamel rods and interprismatic substance. However, the chemical and physical properties for the newly deposited matrix differ from mature enamel. When newly deposited, the enamel matrix is composed of approximately 25 to 30 per cent mineral salts, while the rest is organic material and water. The mature, fully calcified enamel contains 96 per cent mineral salts and only 4 per cent organic constituents and water.

The process of enamel formation is accomplished by ameloblasts depositing a globule of organic matrix one upon the other to form a tall enamel prism which extends from the dentino-enamel junction to the periphery of the crown. Each prism is built up rhythmically and regularly, globule by globule, in daily increments until the ameloblast reaches the tooth periphery. The daily increments are distinguished in the mature enamel by cross-striations (incremental lines of Retzius).

When the enamel prism is completed, it contains calcium salts in only 30 per cent concentration. Final calcification of the enamel matrix begins at the incisal or cuspal tip when the full width of the enamel has been formed and proceeds in planes at right angles to the long axis of the tooth.

Dentinogenesis.—Dentin is formed by cells differentiated from the mesodermal tissue, in contrast to enamel which is formed by cells of ectodermal origin. Dentin formation begins with a deposition of fibrils and matrix at the dentino-enamel junction. Coincident with the formation of these fibrils is the appearance of irregularly spiraling Korff's fibers which originate in the pulp and merge with the fibrils at the dentino-enamel junction. At the same time as the Korff's fibers appear, the spindle-shaped mesenchymal cells closest to the dentino-enamel junction differentiate into tall columnar cells, the odontoblasts. The odontoblasts then form a row of tall columnar cells opposite the tall columnar ameloblasts (C and D in fig. 1). These dentin-forming cells develop processes which extend toward the ameloblasts. As the odontoblasts migrate pulpally, forming as they go incremental layers of dentin matrix (pre-dentin), the protoplasmic extensions of the odontoblasts are left behind within the dentin matrix (the dentinal tubules).

It has been established by numerous studies that, while the ameloblasts and dentin-forming cells deposit matrix, they are extremely sensitive to systemic upsets, toxins, and stress. These systemic disturbances affect the activity of these cells and, in turn, the quality and quantity of matrix deposited. Such defects are permanent and not repairable, so that a history of the health of the animal is inscribed permanently within the structure of the enamel and dentin. These effects can be seen grossly as hypoplastic or hypocalcified defects in enamel (as mottled enamel in fluorosis or hypoplastic defects in the enamel caused by calcium deficiencies or fevers) or as microscopic defects in histologic sections of the tooth such as the neonatal line in the enamel and dentin of the deciduous teeth. Analysis of these lines is termed "tooth ring analysis." This study attempts to delineate, accurately, the exact date at which each level of enamel and dentin is deposited and is therefore a fundamental requirement before tooth ring analysis can be attempted in cattle. It will permit the assessment of the role played by such stress factors as pregnancy, lactation, nutritional deficiencies, and acute infections upon tooth development.

Eruption.—The teeth of cattle, like those of human beings, move from their site or origin within the bone crypt toward their place in the dental arch (E to H in fig. 1). This process of the tooth migration is termed eruption and is a distinct and unique phase in the development of teeth. As will be demonstrated, eruption begins in

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cattle only after all the crown is formed and one third or more of the root is developed (fig. 13). In man, eruption begins within the jaw bone with the formation of the root.[5]

The eruption of teeth occurs in two stages; the prefunctional eruptive phase and the functional phase. The prefunctional phase refers to the tooth movement within the jaws and ends when the teeth occlude with their antagonist in the opposing jaw. The antagonist may be the opposite teeth or the gum pad, as is the case with the lower anterior teeth of cattle. The prefunctional phase of eruptive tooth movement is very complicated. It takes place by three types of movement, incisal toward the occlusal plane, rotatory around a spiral, and lateral toward the midline of the mandible.

Eruption continues even after the teeth are in occlusion (functioning phase). The rate of migration is slow and compensates for the loss of vertical height of the tooth from wear.[6]

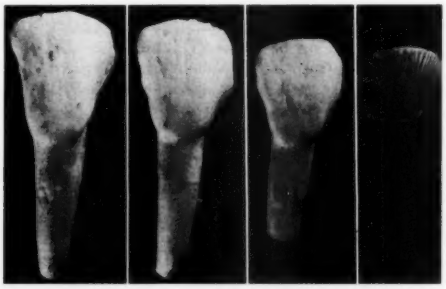

Fig. 3—Anatomy of the three permanent incisors and deciduous canine teeth at different levels of development (dissected from the mandible of a 30-month-old animal). Notice the decreasing size of the crown from the first to the third incisor.

Attrition.—This refers to the normal wear of the enamel and exposed dentin which takes place on the occlusal surfaces of the molars and premolars and on the lingual surface at the incisal tip of the lower anterior teeth. It also takes place on the approximal surface of the teeth. There is a constant slow eruption of the teeth to compensate for the loss of height of the crown caused by wear, to maintain masticatory efficiency. Protection of the pulpal tissue is assured by the continuous deposition of secondary dentin which always takes place in advance of the wearing away process.

The rate of attrition in man is related to his diet, the coarser diets eaten by primitive societies causing a greater amount of wear. Similarly, in cattle, different diets cause different rates of wear. It should be remembered that the mandibular premolars and molars occlude against their equivalent opponents in the maxilla, while the manbidular incisors and canine teeth occlude against the comparatively soft upper gum pad (fig. 2). These differences in occlusion are reflected not only in the rates of wear of the teeth but also by their morphologic characteristics. The molars and premolars have crowns which are long in comparison with their roots, while the incisor crowns are shorter than their roots.

Root Resorption and Shedding.—At birth, the jaws are far too small to support the large permanent teeth, and so the young animal has small deciduous teeth to fit its smaller jaws. When the jaws become larger, the deciduous teeth are shed and replaced in an orderly fashion by the erupting permanent teeth.

Shedding of the deciduous teeth is accomplished by resorption of the root. Associated with this process are osteoclast cells derived from the surrounding connective tissues. In their presence, the cementum and dentin and the surrounding bone are removed by resorption. The destruction usually begins at the apex of the deciduous teeth and proceeds gradually toward the crown. Resorption of bone also takes place around and in advance of the erupting permanent tooth.

Methods

Intra-Oral Radiographs of Incisor Teeth in Cattle

It became obvious that the data required for an accurate study of the chronology of different stages in tooth development could be obtained only through dental radiographs in large numbers of healthy animals whose exact birth dates were known. Fortunately, a method of quickly obtaining accurate intra-oral x rays of the anterior teeth of living cattle had been perfected by Christofferson and Weiss in 1958.[7] The reader is referred to their paper for the details whereby radiographs of the anterior teeth can be obtained quickly and easily in living animals.

Analysis of Radiographs

All the radiographs for the cattle studied were arranged in sequence according to age, by days. Examination was made over a light box with the aid of a magnifying lens (x 5). The deciduous and permanent teeth visible on the radiographs were then analyzed as follows:

A) The deciduous teeth
   1) The amount of root formation at each age period
   2) The amount of root resorption

No material was available to determine the beginning of crown formation *in utero*.

B) The permanent teeth
   1) The beginning and completion of crown formation
   2) The development of the root
   3) The time of intra-osseous eruption
   4) The age at which the tooth emerges into the oral cavity

This last assessment was possible because the level of the incisal edge in relation to the gum margin could be determined in the radiograph.

TABLE 1—Number and Classes of Cattle Studied Radiographically

Breeds*	Birth to 1 year	1 Year to 2 years	2 Years to 3 years	3 Years to 4 years	4 Years to 5 years	Totals for each class
DAIRY						
Holstein-Friesian	62	124	90	76	13	365
Guernsey	4	33	23	15	1	76
Jersey	15	9	5	3	1	33
Brown Swiss	1	1	7	4	1	14
Ayrshire	2	5	0	0	0	7
All Dairy	84	172	125	98	16	495
BEEF						
Hereford	104	68	47	30	9	258
Aberdeen Angus	38	37	18	5	2	100
Shorthorn	8	1	5	1	1	16
All Beef	150	106	70	36	12	374
All Cows	168	262	176	120	22	748
All Bulls	54	14	17	14	6	105
All Steers	12	2	2	0	0	16
Totals for each age group	234	278	195	134	28	869

* Cattle were selected for optimal nutritional status and accuracy of birth date.

It was confirmed by examination of intra-oral color transparencies.

The developmental processes occurred almost simultaneously for each of a pair of incisors, so that they were treated as one. When this was not so, the more advanced tooth was recorded. A difference among members of a pair was most often encountered in the process of eruption.

After the average age and range had been derived for each of the developmental processes in days, the data were regrouped in months to determine the range and average age in months (table 3). The range was finally computed as 1.5 standard deviations of this mean, which included more than 80 per cent of the animals examined and allowed for omission of less than 20 per cent of the very precocious or retarded animals. This was done for each breed separately and, from these, an average and standard deviation for each of the breeds was established (see section on comparison of breeds).

COLOR PHOTOGRAPHS

In addition to the radiographs obtained *in vivo*, a large series of color transparencies of these teeth in living cattle were made immediately after the radiographs were taken. A specially constructed camera with an electronic flash was used. This produced excellent 35-mm. color transparencies for study of the teeth and soft tissues (fig 2).

DISSECTIONS

To supplement the information obtained in the living animals by radiographs and photographs, a number of dried and preserved specimens were dissected to expose the incisor, canine, and molar teeth for the study of three dimensional relationships (fig. 3 and 4).

HISTOLOGIC SECTIONS

Finally, a number of histologic ground and decalcified sections were prepared from selected formalin-fixed specimens. These were studied microscopically to determine more exactly the structural and anatomic relationships of bovine dental tissues and to compare them with the teeth of man (fig. 5 and 6). However, a more detailed analysis and description of the dental and periodontal tissues was left for a subsequent report.

Materials

A total of 869 selected purebred dairy and beef cattle were studied radiographically (table 1). The animals were selected from farms where it would be certain to find optimal nutritional levels and management—and, most important of all, where exact birth dates of the animals would be known.

The beef cattle came from breeding farms and were living under more favored condtions than the usual range animal. Most of the bulls were young and came from beef herds. Most of the dairy bulls were from a large stud farm in Illinois.

Findings

ANATOMIC CONSIDERATIONS

Dental Formulas.—Cattle possess 20 deciduous teeth: three pairs of incisors and one pair of canine teeth in the anterior area of the mandible (none in the maxillae), and three pair of premolars in the posterior areas of the mandibles and maxillae. These are replaced by permanent teeth. The permanent molars do not have deciduous predecessors. The dental formulas are:

$$\text{Deciduous teeth: } (\text{i}\tfrac{0}{3}\ \text{c}\tfrac{0}{1}\ \text{p}\tfrac{3}{3}) \times 2 = 20$$

$$\text{Permanent teeth: } (\text{I}\tfrac{0}{3}\ \text{C}\tfrac{0}{1}\ \text{P}\tfrac{3}{3}) \times 2 = 32$$

The Anterior Teeth.—Many contemporary veterinary authors refer to the fourth anterior tooth of cattle erroneously as a

TABLE 2—Chronology of Root Formation and Resorption in Deciduous Incisor and Canine Teeth

Root formation	Deciduous			
	First incisor (months)	Second incisor (months)	Third incisor (months)	Canine tooth (months)
Root formation completed	6.0 ± 1	6.0 ± 1	6.0 ± 1	7.0 ± 1
Root resorption begins	6.4 ± 1	8.3 ± 2	18.0 ± 3	25.0 ± 3
Resorption advanced*	16.2 ± 1	24.4 ± 1	31.0 ± 1	36.0 ± 1
Exfoliation	23.0 ± 1	30.0 ± 1	35.7 ± 2	42.0 ± 2
Duration of root resorption	16.6	21.7	17.7	17.0

* One third or more of root resorbed.

fourth incisor.[12] This is based on its incisor form and position in the arch. However, studies by Loomis [8] have established that this is a true canine tooth which, since the Oligocene epoch, has slowly changed its canine form and migrated anteriorly to become aligned beside the third incisor. The term "fourth incisor" is incorrect since the Eutheria (placental animals) have only three pairs of incisors and one pair of canine teeth. The evolutionary history of this tooth to its present incisor form and position in cattle is quite clearly shown in paleontologic records.

The calf is usually born with most of its deciduous teeth already partially erupted and visible, to different degrees, within the mouth (fig. 8 and 20). This enables it to partake of a mixed diet of milk and hay shortly after birth. The human child, in contrast, is without any teeth for six months.

The deciduous teeth are replaced consecutively by their permanent successors until the last pair is exfoliated toward the end of the third year. Occasionally a deciduous tooth is retained and, when this occurs, a radiograph will usually show that the permanent successor failed to form.

Occlusion.—The incisor and canine teeth erupt to occlude against the upper gum pad (fig. 6).

Attrition.—The lingual surfaces at the incisal one third of these teeth become abraded early. The enamel is worn away and the underlying dentin is exposed. This takes place at an acute angle to the long axis of the tooth and reflects the curvature of the upper gum pad (fig. 5 and 6). It has been assumed that this abrasive wear is related to the breed of animal and the character of the food ingested.[1] However, the precise mechanism as to how this takes place is still unknown and, in view of its importance to the feeding of these animals, should be investigated in more detail.

Gingivae.—The appearance of the mucosa investing the teeth and covering the supporting bone is similar to that of man. There is a narrow band of free gingiva below which is the pink immovable attached gingiva (fig. 2). The attached gingiva is separated by a distinct muco-gingival junction from the less keratinized, more richly vascular and loosely attached, alveolar mucosa. The latter is reflected onto the inner surface of the lower lip as the muscular mucosa (fig. 2).

Although the external appearance of the gingival tissues are similar to human gingiva, microscopic examination of histologic sections showed that the attached gingiva of cattle is firmly adherent by fasiculi of collagenous fibers to the cementum of the teeth rather than the alveolar bone as in man. A broad mass of connective tissue firmly secures the attached gingiva to the tooth cementum well above the level of the crest of the alveolar bone (fig. 5).

It is commonly thought that the gingivae gradually recede to expose the roots as animals age. This is certainly true in many cases, but not always. Exposure of the roots may occur due to continuous eruption of the teeth. Figure 2 shows the mixed dentition of a 2-year-old animal with the roots of the second deciduous incisor exposed. Comparison with figures 9 and 14 suggests that this eruption occurred because the deciduous anterior teeth would have been below the level of the occlusal plane of the permanent anterior tooth and would become functionless.

Gingivitis is of common occurrence at the time of tooth eruption and appears similiar to the eruption gingivitis described in children.[9] Quite often, traumatic gingivitis is observed, caused by fragments of trapped vegetation.

Incisor Tooth Form.—The first permanent incisor has the largest crown and the longest root. The crowns have been likened to shovels, whose proximal surfaces are flared. The second and third incisors are successively smaller (fig. 3). The canine tooth is incisor in form and often appears to be larger than the third incisor. This

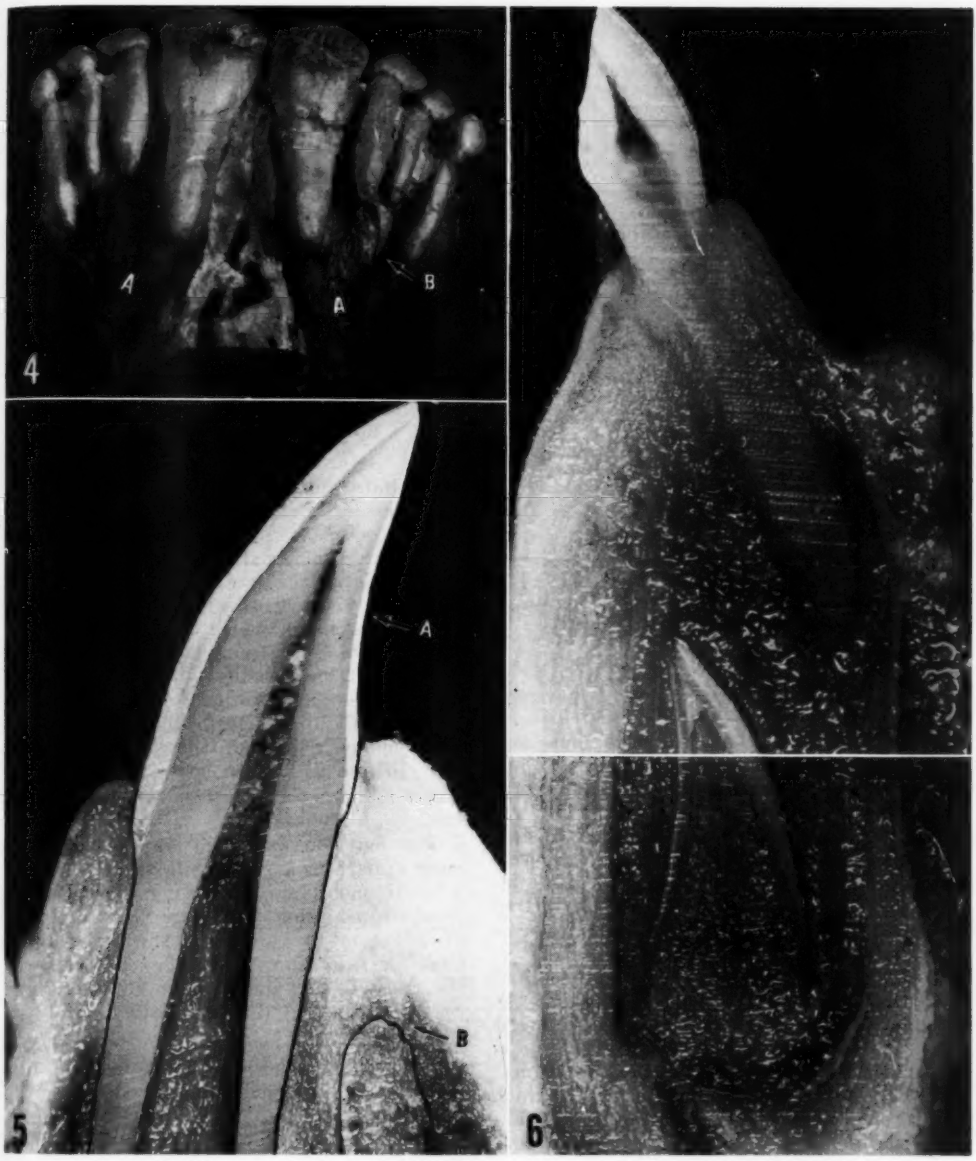

Plate I

Fig. 4—Mandible from a 22-month-old animal dissected to show the relation between the erupted deciduous teeth and the developing permanent tooth germs. The root of the permanent first incisor is still incomplete. The second permanent incisor (A) lies lingual to the root of the first, while the third permanent incisor tooth germ (B) can be partly seen lingual to the crown of the second incisor.

Fig. 5—A buccolingual section through the mandible showing a recently erupted first permanent incisor. Notice the width of the labial enamel and the thin lingual enamel (A), also the low level of the crest of the alveolar bone (B). The marginal gingivae are attached to the cementum, instead of the bone, by dense collagenous fibers. The parallel markings on the dentin are from saw cuts.

Fig. 6—A labiolingual section showing the relation of the developing permanent incisor to the lingual apical surface of the deciduous predecessor.

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TABLE 3—Chronology of Development of Permanent Incisor and Canine Teeth

Development	Permanent			
	First incisor (months)	Second incisor (months)	Third incisor (months)	Canine tooth (months)
Crown formation begins	6.0	12.2 ± 1	20.0 ± 1	27.5 ± 2.5
Crown formation completed	12.3 ± 1	18.6 ± 1	24.0 ± 1	33.4 ± 2
Intra-osseus eruption begins	18.2 ± 1	24.7 ± 1	30.3 ± 2	38.0 ± 2
Emergence into oral cavity	23.0 ± 1	30.0 ± 1	35.7 ± 2	42.0 ± 2
Root formation completed	28.0 ± 1	35.0 ± 2	43.5 ± 2	52.0 ± 2
Duration of crown formation	6.3	6.4	4.0	5.9
Duration of intra-osseus eruption	4.8	5.3	5.4	4.0
Duration of root formation	15.7	16.4	19.5	18.6

is equally true for the deciduous and permanent incisors.

When the teeth first appear in the oral cavity, the enamel is marked by several vertical striations which become polished and erased by age and wear.[1] These vertical striations are so large that it is unlikely that they reflect the corrugated contour of the dentino-enamel junction as stated by Garlick.[3]

Enamel and Dentin.—A longitudinal section through the crown shows that the enamel cap is thick and even on the labial surface, but much thinner on the lingual surface (fig. 5). The enamel is thinnest at the incisal one third of the tooth which probably accounts for the rapid exposure of the dentin in this area once the tooth has started to function.

The dentin extends practically the whole length of the tooth. In the crown it is covered by a cap of enamel, and in the root it is protected by a thin layer of cementum. The cementum usually overlays a small portion of the cervical border of the enamel. The cementum is the specialized tissue which embeds the fibers of the periodontal ligament and thus secures the tooth within its bony socket.

The central cavity of the dentin is filled with a sensitive soft tissue, the pulp. In the young animal, the pulp is large and vascular, but as the tooth becomes abraded, the odontoblasts that line the pulpal wall are stimulated to deposit secondary dentin until, in the older animal, the pulp of the tooth is nearly filled in.

The Attachment Apparatus.—The incisor and canine teeth in cattle have a somewhat different attachment apparatus than those in man. When the tooth has reached its functional level, only the apical two thirds of the tooth lies within bone, while the cervical third is supported by collagenous fibers which run between the cementum and the gingivae (fig. 5). The periodontal membrane is wide and there is no dense lamina dura of bone as is found in all human teeth and in the premolar-molar region of these same cattle (fig. 16 and 17). The bone of the labial aspect of the alveolar process is thinner than the lingual bone.

The loose arrangement of the attachment apparatus may explain why the tooth is quite mobile in the living animal. It would appear that this is another example of adaption to function. The wide and loose periodontium may serve to cushion the effect of the incisal edge striking the premaxillary pad during mastication.

Relation of Permanent Tooth Germ to Deciduous Incisor and Canine Teeth.—As in man, the tooth germs of the permanent incisor and canine teeth are derived from the lingual aspect of the enamel organ of the corresponding deciduous tooth. The permanent incisor and canine tooth germs, therefore, come to lie in the region of the

TABLE 4—Chronology of Development of Permanent Mandibular Premolar and Molar Teeth

Development	Permanent					
	First premolar (months)	Second premolar (months)	Third premolar (months)	First molar (months)	Second molar (months)	Third molar (months)
Crown formation begins	12–13	9–10	11–12	In utero	1	9–10
Crown formation completed	22–23	16–20	24–30	2–3	12–13	23–24
Root formation completed	36–40	36–40	36–40	13	24–25	38

deciduous root apex and lingual to it (fig. 6 and 16).

The large developing permanent incisors are crowded within the small, still growing jaw. They cannot align themselves correctly within this space and, therefore, become staggered and rotated. The second permanent incisor lies lingual to the root of the first, while the third incisor tooth germ can be seen just behind the crown of the developing second incisor (fig. 4). When the teeth first emerge they are unevenly placed, but they soon become correctly aligned by the moulding action of the muscular tongue and lips. This has been adequately described in children.[10] The mechanism in cattle is probably similiar.

Chronology of Incisor and Canine Tooth Development

DECIDUOUS ANTERIOR TEETH

Initiation.—Garlick [3] has indicated that initiation of proliferation of epithelial cells from the oral epithelium takes place sometime during the second month of intrauterine life. However, his study was not a chronologic one and additional work on the chronology of tooth development in utero is needed.

Beginning Crown Formation.—The advanced stage of the deciduous incisor tooth development at birth indicates the deposition of enamel and dentin matrix begins approximately during the fourth to fifth month *in utero* and possibly earlier.

Completion of Crown.—The crowns of the deciduous incisors are probably completely formed two to three months after the beginning of enamel formation—that is, at seven to nine months *in utero* from the first to the fourth incisor.

Eruption.—Emergence of the teeth into the oral cavity takes place at birth and shortly afterward (table 6). The first, second, and third deciduous incisors may have a quarter or more of their crowns exposed in the oral cavity at birth. The

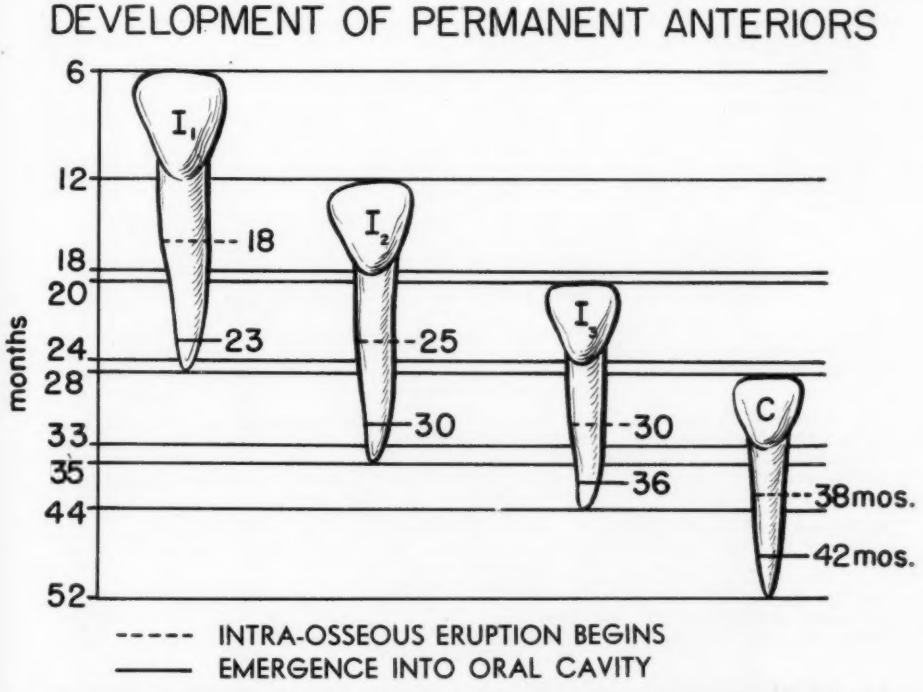

Fig. 7—A composite drawing showing the chronologic sequence in the development of the three permanent incisor and the canine teeth showing the beginning and completion of crown formation, level of root formation at beginning of intra-osseous eruption and age of emergence into the oral cavity.

canine teeth will appear when the animal is approximately 2 weeks old.

Root Formation.—At the time of the emergence, the anterior teeth have from one half to two thirds of their roots formed (table 5). Completion of the roots takes place approximately 6 months of age for the first three incisors and one month later for the canine teeth (table 2).

Resorption.—Resorption of the first deciduous incisor starts soon after the root has formed and is coincident with the appearance of the first permanent incisor follicle at 6 months (tables 2 and 5). The second incisor begins to resorb only a little later, and this seems to be due to the proximity of the large first permanent incisor. Resorption of the third incisor and canine teeth (like the first incisor) is coincident with the appearance of their permanent successor tooth germs (tables 2, 3, and 5).

Exfoliation.—Loss of the deciduous tooth takes place only slightly before the permanent successor emerges into the oral cavity (fig. 14 and table 5). Usually, three quarters or more of the root must be resorbed before exfoliation can take place (fig. 13).

PERMANENT INCISOR AND CANINE TEETH

Initiation.—The exact age for the beginning of tooth bud formation cannot be determined from radiographs, as the density of the surrounding bone masks the earliest tissue proliferations. However, the first radiolucency indicating the tooth follicle has been shown histologically to represent a tooth germ already well advanced toward the bell stage of tooth development, with matrix deposition ready to begin (fig. 9). Additional histologic analysis will be necessary to determine the chronology of this phase of development.

Crown Formation.—Enamel and dentin formation begin with the deposition of layer upon layer of enamel and dentin matrix by the ameloblasts and odontoblasts. After the matrix is deposited, calcification takes place; there is a lag between matrix deposition and its calcification in the enamel. Therefore, the chronology of enamel development as derived from radiographs will be slightly later than when it is derived from histologic sections.

The three permanent incisor and canine teeth begin to form at 6, 12, 20, and 27 months, respectively (table 3). The crowns of the first incisor, second incisor, and canine teeth are completed six months later, while the third incisor forms more rapidly, being completed only four months later (fig. 7).

Rate of Permanent Crown Formation.—The crown of the first incisor, second incisor, and canine teeth require about six months to form, while the third incisor

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Legend for Figures 8-12 (Plate 2) on Opposite Page

Fig. 8—Radiograph of the distal extremity of a dried mandible at birth. All crowns of the deciduous teeth are complete and one half or more of their roots are formed. Each appears at different levels of eruption. The midline separation is an artefact. (All the radiographs in this series are actual size).

Fig. 9—Radiograph of teeth of a 6-month-old female Holstein-Friesian. The deciduous incisors are completely formed. The tooth germs of the first permanent incisors are apparent as circumscribed radiolucent areas at the apices of the first deciduous incisors (A). The mental foramen lies below the developing tooth germ and appears as a smaller more sharply defined radiolucent area (B).

Fig. 10—Radiograph of teeth of a 10-month-old Holstein-Friesian heifer. Two thirds of the crown of the first permanent incisor is formed. The arrow indicates the crest of the alveolar process.

Fig. 11—Radiograph of teeth of a 12-month-old Hereford female. The crown of the first permanent incisor is almost completed and the crypt of the second incisor is visible just below the apex of the second deciduous incisor (B). Resorption of first deciduous incisor indicated by (A).

Fig. 12A and 12B—Radiographs of a preserved specimen from an 18-month-old cross-bred Holstein-Friesian; (A) labiolingual view, (B) lateral view of one half of the jaw showing the alignment of the first permanent incisor crown at right angles to the other teeth and the lingual position of the second incisor. The arrows indicate areas of advanced resorption on the lingual surface of the first deciduous incisor.

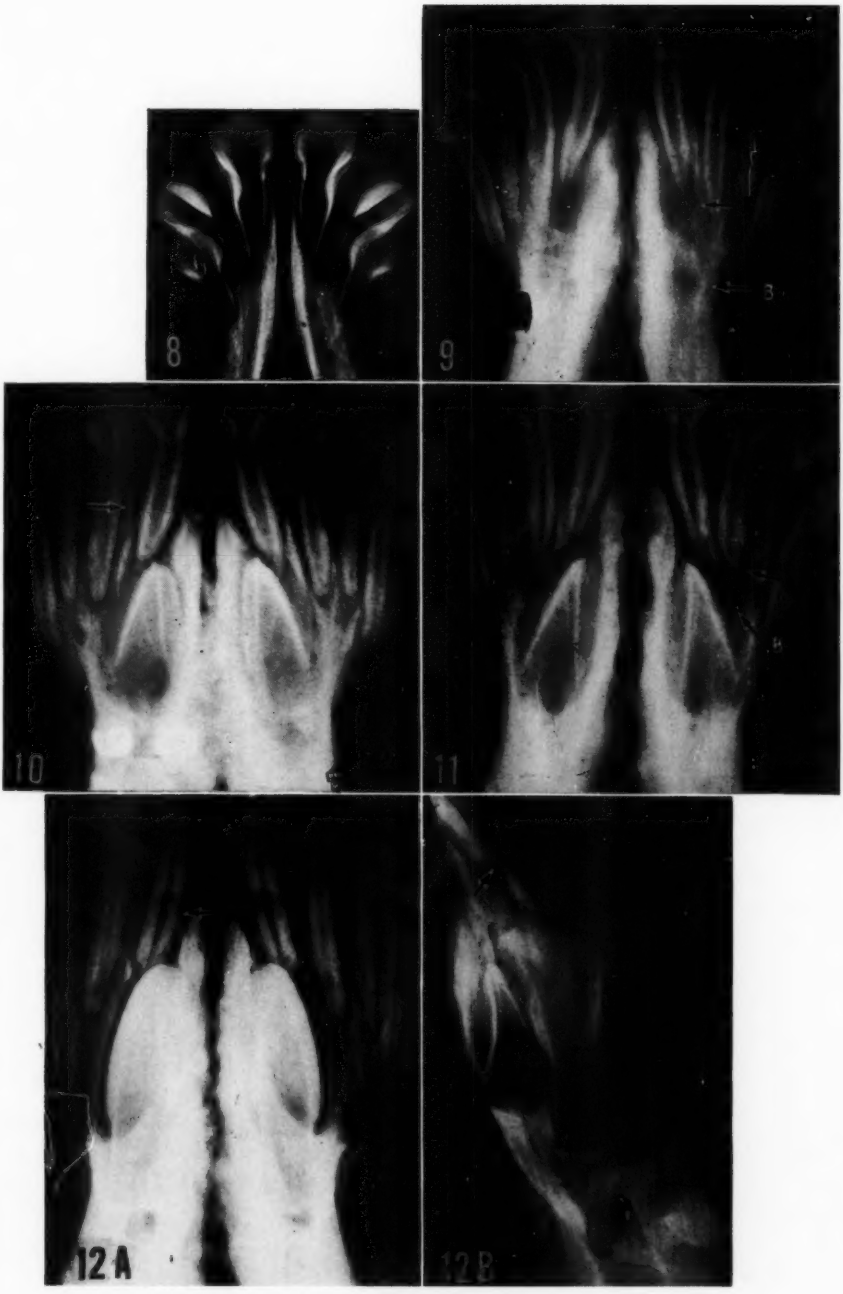

Plate 2

TABLE 5—Composite Chronology of Development of Mandibular Teeth* (Anterior)

	Deciduous				Permanent			
	First incisor	Second incisor	Third incisor	Canine tooth	First incisor	Second incisor	Third incisor	Canine tooth
Bir.h	¾ erupted—root ⅔ formed	⅔ erupted—root ⅔ formed	½ erupted—root ½ to ⅔ formed	¼ erupted—root ½ formed	Absent	Absent	Absent	Absent
½–1 yr.	Root formation completed—root resorption begins	Root formation completed—root resorption begins	Root formation completed	Root formation completed	Crown formation begins	.....	.....	.....
1–1½ yr.	Root resorption advanced	.....	.....	.....	Crown formation completed	Crown formation begins	.....	.....
1½–2 yr.	Exfoliated	.....	Root resorption begins	.....	Intra-osseous eruption begins	Crown formation completed	Crown formation begins	.....
2–2½ yr.	.....	Root resorption advanced	.....	Root resorption begins	Emergence into oral cavity—root formation completed	Intra-osseous eruption begins	Crown formation completed	Crown formation begins
2½–3 yr.	.....	Exfoliated	Root resorption advanced	.....	.....	Emergence into oral cavity	Intra-osseous eruption begins	Crown formation completed
3–3½ yr.	.....	.....	Exfoliated	Root resorption advanced	.....	Root formation completed	Emergence into oral cavity	Intra-osseous eruption completed
3½–4 yr.	.....	.....	.....	Exfoliated	.....	.....	Root formation completed	Emergence into oral cavity
4–4½ yr.	.....	.....	.....	.....	.....	.....	.....	Root formation completed

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TABLE 5—(Continued) Composite Chronology of Development of Mandibular Teeth* (Posterior)

	Deciduous premolars			Permanent premolars			Permanent molars		
	First premolar	Second premolar	Third premolar	First premolar	Second premolar	Third premolar	First molar	Second molar	Third molar
Birth	⅓ erupted	⅔ erupted	¾ erupted	.....	.....	.....	⅓ of crown formed—completed at 2–3 mo.	Crown formation begins at 1 mo.	.....
	All roots complete 1–2 mo. later								
½–1 yr.	.....	.....	.....	.....	Crown formation begins	Crown formation begins	Emergence into oral cavity	Crown formation completed	Crown formation begins
1–1½ yr.	.....	.....	.....	Crown formation begins	Crown formation completed	.....	Root formation completed	Emergence into oral cavity	.....
1½–2 yr.	Root resorption present to different degrees			Crown formation completed	.....	Crown formation completed	.....	.....	Crown formation completed
2–2½ yr.	Exfoliated			.....	Emergence into oral cavity	.....	.....	Root formation completed	Emergence into oral cavity
2½–3 yr.	Exfoliated	.....	Exfoliated	Emergence into oral cavity	.....	Emergence into oral cavity	.....	.....	.....
3–3½ yr.	.....	.....	.....	Root formation completed	Root formation completed	Root formation completed	.....	.....	Root formation completed

* Based on analysis of intra-oral radiographs of 869 living cattle selected for optimal nutritional status and accurate birth dates.

crown is completed in four months. The crown of the third incisor is smaller than the first and second incisor (fig. 3) and observation has shown that the canine tooth often appears larger and slightly different than the other three. This may be correlated with the fact that the fourth anterior tooth is in reality a canine tooth and only in the Oligocene era has come to have its present incisor-like form and position.[8]

The initiation of the first incisor crown formation lies within a narrow range of time (table 3). The second and third incisors have a larger range, while the permanent canine teeth may start development anywhere between 25 and 33 months.

The range of ages over which the crown of the first three incisors are completed are quite similar but the range is doubled for the canine teeth.

Intra-Osseous Eruption.—When a third or more of the permanent incisor roots are formed, migration within the mandibular bone begins. It commences for the first permanent incisor at 18 months and for the second incisor, third incisor, and canine teeth at 25, 30, and 38 months, respectively (table 3).

Emergence into the Oral Cavity.—Emergence into the oral cavity occurs when approximately four fifths of the root has been formed (fig. 7). This usually takes place approximately four to five months after intra-osseous eruption began.

Rate of Eruption.—Eruption commences when a third or more of the root is formed and it takes from four to five and one half months for each of the teeth to emerge into the oral cavity. However, eruption does not end with the appearance of the incisor into the oral cavity nor when it strikes the upper gum pad. Eruption still proceeds, but very slowly, to compensate for crown wear.

Table 3 indicates a greater range in the time and rate of eruption for the third incisor and canine teeth than for the first and second incisors.

It will be apparent from the foregoing description of these processes, that there is a tendency for an increasing range in the ages at which they occur—from the first incisor to the canine in tooth sequences, and from the beginning of crown formation to the completion of root formation in time sequence.

Root Formation.—Root formation begins when the crown is completely formed. It is not completed until the tooth has been in active function within the oral cavity for approximately five months for the first and second incisors and nine months for the third incisors and canine teeth (table 3).

Rate of Root Formation.—The root takes from 15.7 to 19.5 months to form (table 3) in contrast to the six years required for human tooth root formation.[9] The range is larger for the second and third incisor

Legends for Figures 13-18 (Plate 3) on Opposite Page

Fig. 13—Radiograph of teeth of a 20-month-old Holstein-Friesian, the arrow indicates area showing the first evidence of calcification for the third incisor behind the crown of the second incisor. Root formation has begun for the second incisor. The first permanent incisor has started to rotate and erupt and resorption of the root of the first deciduous incisor is well advanced.

Fig. 14—Radiograph of teeth of a 23-month-old Holstein-Friesian heifer. The first incisor on the left is about to erupt into the oral cavity. Its rotation is nearly completed. The deciduous first incisors are ready to exfoliate.

Fig. 15—Radiograph of teeth of a 27-month-old Holstein-Friesian heifer. Arrow indicates area showing beginning calcification of the canine tooth lingual to the crown of the third incisor. The crown of the third incisor is completed and its root has started to develop. The first incisor has erupted into the oral cavity.

Fig. 16—Radiograph through one half of a mandible from an animal slightly younger than the one shown in figure 15. Notice the spatial arrangement of the three developing permanent incisors.

Fig. 17—Radiograph of teeth of a 3-year-old Holstein-Friesian cow. The third permanent incisor is about to emerge into the oral cavity as soon as the deciduous tooth is exfoliated. The root of the first and second permanent incisors are completed.

Fig. 18—Radiograph of teeth of a 4½-year-old Holstein-Friesian cow. The permanent dentition is complete.

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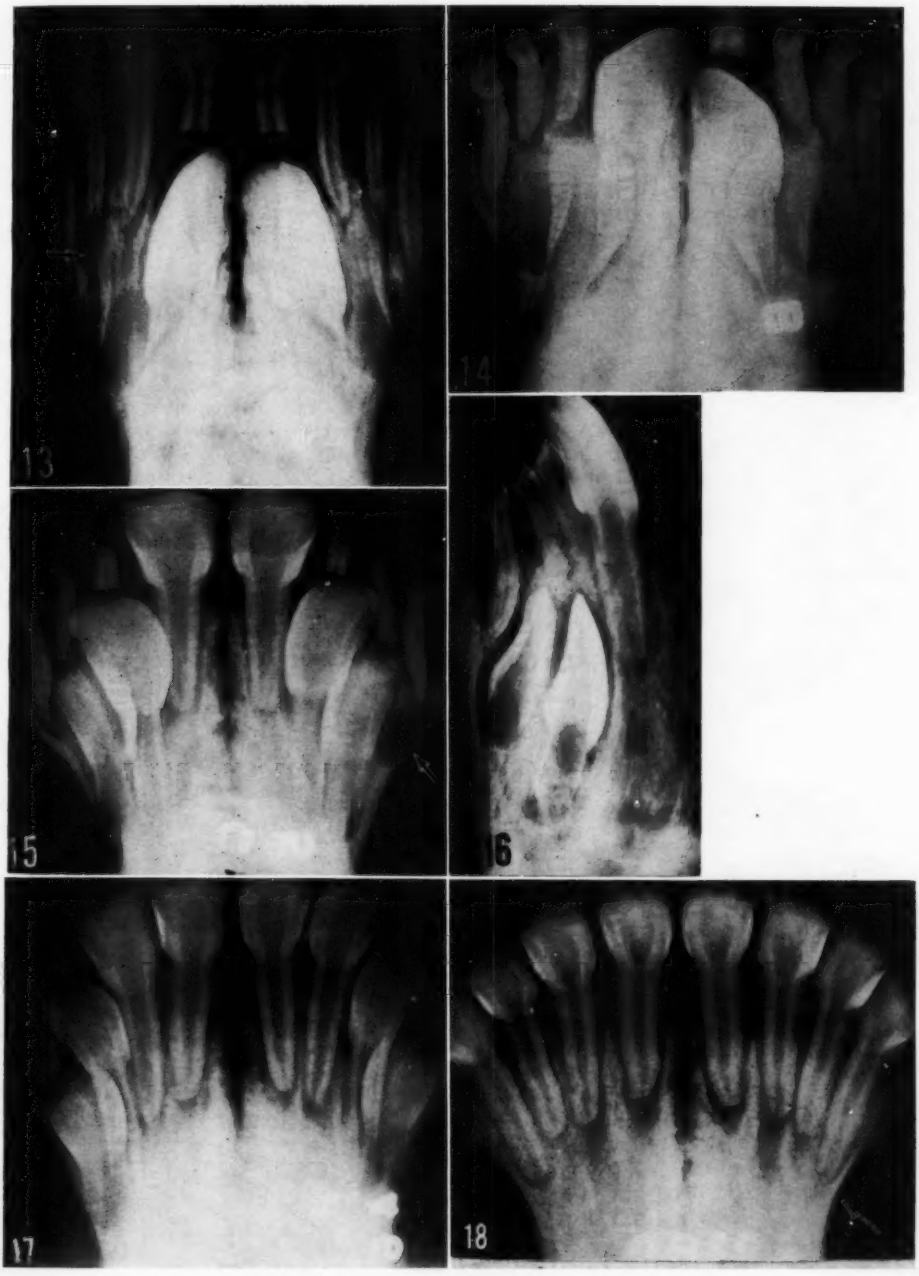
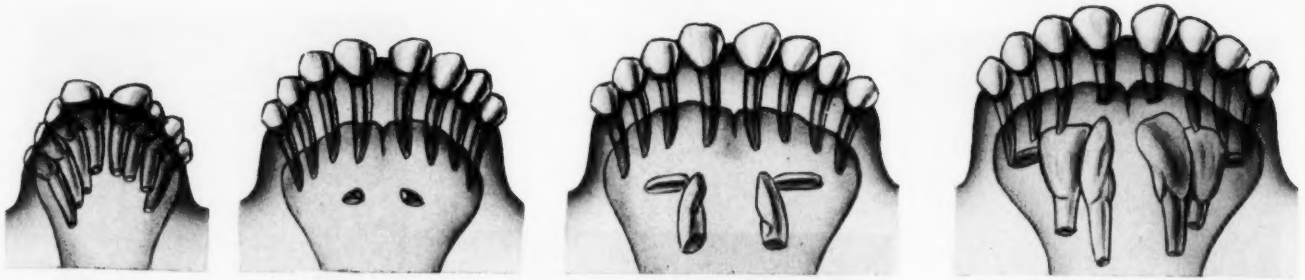


Plate 3



Birth

6 months

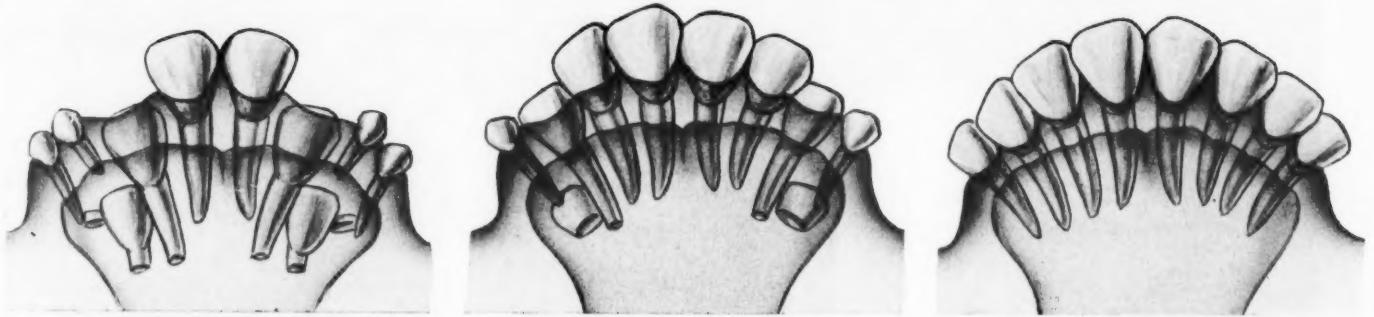
I_1 Crown begins

1 year ± 1 mo.

I_1 Crown completed
I_2 Crown begins

$1\frac{3}{4}$ years ± 1 mo.

I_3 Crown begins



$2\frac{1}{4}$ years ± 2 mos.

C Crown begins
I_1 Root completed

$2\frac{3}{4}$ years ± 2 mos.

C Crown completed
I_2 Root completed

$4\frac{1}{4}$ years ± 2 mos.

Anterior teeth completed

Figure 19

[22]

than it is for the first. There was inadequate data to show the frequency and distribution for the canine tooth, the estimate for completion at 52 ± 2 months being made from 28 animals ranging from 4 to 5 years of age.

Stages in Incisor and Canine Tooth Development

Birth

When the calf is born, nearly all the deciduous teeth have erupted, to different degrees (table 5 and fig. 8). The first incisor is usually fully erupted but the canine teeth may still be unerupted. Radiographs show that one half or more of the roots are formed and the pulp canals are still wide (fig. 8), but they show no signs of the permanent successors.

The jaws at birth are not large enough to accommodate the deciduous teeth, so the crowns and the roots are rotated and overlap each other (fig. 8). As the jaws enlarge, this crowding is relieved (fig. 10).

Six Months

This age is characterized by the appearance of the tooth germ of the first permanent incisor in the radiograph (fig. 9). The earliest evidence of permanent incisor tooth development is the appearance of a circumscribed radiolucent area at the level of the first deciduous incisor. The radiographs do not show the calcified enamel at this stage, as calcification is of insufficient density to be contrasted with the investing bone. Below the permanent incisor follicle is another, but smaller, circular radiolucent area. This is the foramen for the mental nerve.

In the meantime, the roots of the deciduous incisor have been completed and a marked decrease in pulpal width is also apparent. The deciduous tooth roots begin to be resorbed with the appearance of the first permanent tooth follicle. Resorption starts in the apical region and, from there, spreads in an orderly manner up the length of the root until the tooth is exfoliated. Resorption is recognized on a radiograph by small areas of varying radiolucency in the dentin.

Periodontium.—The nature of the attachment of the tooth to the jaw is now apparent in the radiograph (fig. 9). A quarter or more of the root of the developing deciduous tooth has erupted beyond the crest of the alveolar process. The part of the root lying within bone is attached to the latter by the periodontal membrane, distinguished in the radiograph as a dark radiolucent band around the tooth root.

Ten Months

This age is characterized radiographically by two thirds of the crown of the permanent first incisor being completed (fig. 10). The crown lies at right angles to the plane of ultimate alignment in the mouth as shown at 18 months (fig. 12A and 12B).

The Deciduous Incisors.—The radiograph shows a progressive reduction of crown size due to attrition and a narrowing of the pulp due to secondary dentin formation (fig. 10). Half of the incisor root now lies above the alveolar crest due to continued eruption of these teeth. As previously stated, this area of the root is supported by thick bundles of collagenous fibers which are covered by the attached gingivae. There is no evidence, clinically, of gingival recession. The periodontal membrane lies between the cementum covering the root and the alveolar bone which lines the tooth socket. This membrane (or ligament as it is sometimes called) is quite wide especially around the first and second deciduous incisors.

Twelve Months

Calcification of the permanent second incisor crown begins at 1 year, at the same time that the crown of the first incisor is completed.

The Deciduous Teeth.—Areas of resorption are well defined on the root of the first deciduous incisor (fig. 11). Early signs of resorption can also be seen on the root of the second incisor. Resorption of the second deciduous incisor begins sometime before the appearance of the follicle for the second permanent incisor due to im-

Legend for Figure 19 on Opposite Page

Fig. 19—Composite drawings showing the various stages of the incisor and canine tooth development at different ages.

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TABLE 6—Composite Chronology of Mandibular Tooth Development

Teeth	Crown formation begins	Age of eruption	Sequence of development
DECIDUOUS			
1st incisor	in utero*	0– 7 days	1
2nd incisor	in utero	0–14 days	2
3rd incisor	in utero	0–21 days	3
Canine tooth	in utero	14–42 days	4
1st premolar	in utero	0–14 days	3
2nd premolar	in utero	0–10 days	2
3rd premolar	in utero	0–10 days	1
PERMANENT			
1st incisor	6 mo.	23 ± 1 mo.	3
2nd incisor	12 ± 1 mo.	30 ± 1 mo.	6
3rd incisor	20 ± 1 mo.	36 ± 2 mo.	8
Canine tooth	27.5 ± 2 mo.	42 ± 2 mo.	9
1st premolar	13 mo.**	24–30 mo.†	7
2nd premolar	10 mo.	18–30 mo.	4
3rd premolar	11 mo.	30–36 mo.	5
1st molar	in utero	5– 6 mo.	1
2nd molar	1 mo.	12–18 mo.	2
3rd molar	10 mo.	24–30 mo.	4

* Data not available. ** Standard deviation not determined. † Premolar and molar eruption age after Sisson, 1953.

pingement of the crown of the first permanent incisor upon the roots of the second, as well as the first, deciduous incisor. As seen in figure 4, the partly formed third permanent incisor lies behind the crown of the second permanent incisor.

The Permanent Incisor Teeth.—Development of the first permanent incisor proceeds rapidly. The crown is completely formed in six months.

The tooth germ of the second permanent incisor begins to deposit enamel and dentin within the dental follicle. This tooth lies lingual to the first incisor tooth crown, and is located at the apex of the second deciduous incisor (fig. 11).

TWENTY MONTHS

At 20 months, the third incisor begins to calcify and the second incisor is completed.

The Deciduous Incisor Teeth.—Resorption of the root of the first deciduous incisor is well advanced at 20 months due to the continued intraosseous movements of the first permanent incisor (tables 2, 3, and 5).

Root resorption may be visualized as a biphasic process: (1) resorption at the apex of the root in response to the proximity of a developing tooth germ and (2) advanced semicircular resorption in response to the permanent successor's eruptive movements. In the latter phase of resorption, osteoclasts ascend along the lingual and mesial surfaces of the root on a broad semicircular front. This is better understood by comparing the two radiographs taken at right angles to each other as shown in figures 12A and 12B.

The labial surface of the root is the last to become resorbed. Sometimes it does not become resorbed at all and causes a lingual deflection of the erupting permanent incisor.

The Permanent Incisor Teeth.—By the 20th month, half of the root of the first incisor is completed (fig. 13). It has already begun its intraosseous eruption. Apparently eruption begins when approximately one third to one half of the root is completed.

The crown of the second incisor is completed and its root is just beginning to form. The outline of the follicle for the third incisor appears behind this crown near the root apex of the third deciduous incisor. This represents a stage two or three months in advance of the dissected specimen shown in figure 4. Three months later, at the age of 23 months, the right incisor is slightly in advance of the left (fig. 14). A slight difference in eruption is often observed between the two teeth of the pair. Also, the right permanent first incisor has rotated a quarter turn during its migration.

TWENTY-SEVEN MONTHS

This age is characterized by the beginning of calcification of the canine tooth.

TABLE 7—Chronology and Order of Tooth Eruption in Cattle

	Deciduous dentition	5 mos. ⟷		6 mos. ⟷		6 mos. ⟷		6 mos. ⟷		6 mos. ⟷		6 mos. ⟷	
	i_1 i_2 i_3 p_3 p_2 p_1 c		M_1		M_2		I_1		P_2 M_3	I_2	P_3 P_1	I_3	C
Age	0–½ mo.	½–1 mo.	6 mos.		12 mos.		25 ± 1 mos.			30 ± 1 mos.		35 ± 2 mos.	42 ± 2 mos.
Crown formation begins	in utero		in utero		1 mo.		6 mos.		10 mos. 10 mos.	12 mos.	11 mos. 13 mos.	21 mos.	27 mos.

The crown of the third incisor was completed at 24 months. Meanwhile, the first incisor has erupted and, clinically, the mixed incisor dentition period has begun (fig. 2).

The Deciduous Incisor and Canine Teeth —The pulp canals of these deciduous teeth are practically obliterated and only portions of the crowns remain (fig. 15). The roots often are apparently extruded above the level of the gum margin and worn quite a bit by the abrasive action of grass and other feed being pulled between the teeth (fig. 2).

The first deciduous incisor was exfoliated when the first permanent incisor erupted three months previously. The root of the second deciduous incisor is almost entirely resorbed and the tooth is very mobile.

Resorption of the apices of the third incisor and canine teeth is well advanced and a small amount of bone supports these deciduous teeth (fig. 15).

The Permanent Incisor and Canine Teeth. —The first incisor took its place within the oral cavity at 2 years of age when four fifths of the root was formed. Now, the root is complete.

Three quarters of the root length of the second incisor is completed and intraosseous eruption is well advanced. In three

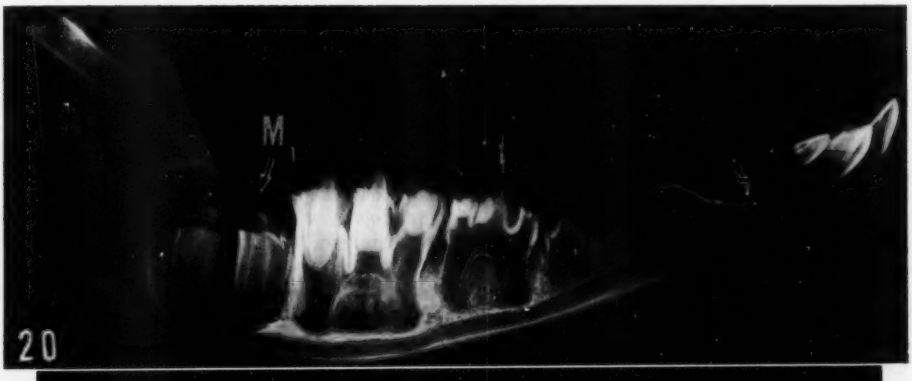

Fig. 20—Radiograph of a mandible of calf at birth showing relation of the deciduous incisors and premolars. The first deciduous incisor and third deciduous premolar are the first to emerge followed by the second incisor and the second premolar, then the first deciduous premolar and third deciduous incisor. The canine tooth is last to erupt. The developing crown of the first permanent molar (M_1) and the mandibular canal are visible.

Fig. 21—Radiograph of a 2-month-old bull's mandible. The three deciduous premolars are fully formed and are in functional occlusion. The crown of the first permanent molar is complete with a quarter of the root formed. The outline of the second molar follicle is seen with a third of its crown formed. The lamina dura shows around the roots of these teeth as compared with its absence around the anterior teeth.

months, the tooth will emerge into the oral cavity.

One third of the root of the third incisor has formed, but there is as yet no evidence that the tooth has commenced to erupt. Its incisal edge still lies below the apex of its permanent successor.

The tooth follicles of the canine teeth are apparent in the radiograph, indicating the beginning of crown formation. In some cases, calcification at the incisor edge can just be detected (fig. 15).

A radiograph at a slighty earlier age through a half section of a mandible (fig. 16) demonstrates the spatial arrangement of the permanent anterior teeth and shows how they each lie one behind the other within the limited space of the bony mandible.

The Arch Form.—From the earliest stages of development, with the eruption of the deciduous anterior teeth into the oral cavity, there has been a gradual enlargement of the mandibular complex which supports these teeth. In the beginning, the teeth were tightly packed together, but during the next two years, they have erupted farther into the oral cavity and spread out to form a smooth arch. Now, at 27 months, the crowns have become separated, partly from the wearing away of their broader incisal tips, but also because the eruptive movements are on a gentle curve carrying the teeth away from the midline.

This is part of the widening process of the mandible which allows for the development of the permanent teeth and, when they erupt, assures them adequate space to function.

THREE YEARS

The eruption of the third permanent incisor and exfoliation of its deciduous predecessors occurs at 3 years of age. Only a small part of the deciduous roots of the canine teeth are supported by bone (fig. 17).

The Permanent Incisor and Canine Teeth.—The first and second incisors have their roots completed. The third incisors are also erupted with four fifths of their roots formed. The canine tooth is not yet erupted but one third (or slightly more) of its root length is completed and it will shortly begin to move toward the oral cavity (fig. 17).

The Periodontium.—Until this stage is reached, it is not possible to see the periodontal membrane clearly, because of the overlapping teeth. The cervical third of the root of the first and second incisors are above the alveolar crest. The apical two thirds of the roots are embedded in bone. The roots are secured to the bone by collagenous fibers which form the periodontal membrane. This is seen radiographically as a narrow radiolucent band running between the root and the investing bone. It appears to be especially wide at the apex of the tooth (fig. 17). This probably represents an area of grouped collagen fibers which contributes to the cushioning of the tooth as it strikes against the upper gum pad.

FOUR AND A QUARTER YEARS

All the permanent incisor and canine teeth and their roots are completely formed (fig. 18).

TABLE 8—Chronology of Tooth Development in Different Breeds of Dairy and Beef Cattle

		A. Comparison of beginning crown formation				B. Comparison of completion of crown formation			
Breeds	No. animals	First incisor (months)	Second incisor (months)	Third incisor (months)	Canine tooth (months)	First incisor (months)	Second incisor (months)	Third incisor (months)	Canine tooth (months)
Dairy and beef	869	6.0	12.2 ± 1	20.0 ± 1	27.5 ± 2.5	12.3 ± 1	18.6 ± 1	24.0 ± 1	33.2 ± 2
Dairy	495	6.0	12.7 ± 1	19.4 ± 1	27.3 ± 2	12.0 ± 1	18.0 ± 1	24.0 ± 1	33.4 ± 2
Holstein-Friesian	365	6.0	11.8	19.5	26.5	11.6	17.4	24.0	33.0
Guernsey	76	....	14.0	19.0	27.6	13.0	18.0	24.7	33.0
Jersey*	33	....	.....	19.0	.....	.....	.....	24.0	34.0
Brown Swiss*	14	6.0	12.0	19.0	27.6	.....	17.0	.....	33.0
Ayrshire	7	....	13.0	21.0	.....	.....	19.0	.....	.....
Beef	374	6.0	11.6 ± 1	20.5 ± 1	27.8 ± 2	12.5 ± 1	19.2 ± 1	24.0 ± 1	33.0 ± 2
Hereford	258	6.0	11.4	20.5	28.8	13.0	20.0	24.5	32.5
Aberdeen Angus	100	6.0	11.7	19.5	26.8	11.5	17.0	23.0	31.0
Shorthorn*	16	6.0	.....	.....	.....	13.0	.....	.....	.....
Bulls (all breeds)	105	6.0	12.0 ± 1	.....	27.4 ± 2	12.6 ± 1	18.0 ± 1	26.0 ± 1	.....

A complete summary of these stages of incisor and canine tooth development is illustrated by composite drawing (fig. 19).

Comparison of Tooth Development in Different Breeds

There are marked physical, physiological, and developmental differences between the dairy and beef breeds. They attain body maturity at different ages and reach mature weights and heights at different ages. In addition, the body economy of each is geared toward different objectives—the dairy animals to produce milk and the beef animals to acquire flesh. Because of the different economies, farm management is based on different principles.

Furthermore, the breeds are individual types with different characteristics. It was, therefore, thought necessary to test whether the chronology of tooth development differed among the different breeds and sexes.

METHOD OF ANALYSIS

The radiographs for all the dairy cattle were first arranged in sequence according to age, disregarding the different types or breeds of animals within this group. The same was then done for all the beef animals. From these radiographs, the mean and 1.5 standard deviations were then calculated for the following stages in the development of each of the incisors and the canine teeth:

1) Beginning and completion of crown formation
2) Eruption into oral cavity
3) Completion of root formation

The chronology of tooth development was then compared for all dairy and for all beef cattle (fig. 25).

When this was completed, the same data were computed and compared for the Holstein-Friesian, Guernsey, Hereford, and Aberdeen Angus breeds separately. There were inadequate data to compare on an equivalent basis the Jersey, Brown Swiss, and Shorthorn breeds, but by extrapolation it was possible to obtain some indication as to whether the chronology of their tooth development was similiar or differed widely from the other breeds. The similarity of the chronology of tooth development among all the breeds studied, individually or collectively, is shown (table 8).

Finally, the chronology of tooth development was computed for all the bulls and compared with chronology of tooth development for the combined beef and dairy herds.

The findings in detail were as follows:

Beginning of Crown Formation.—The first incisor started to form for all the breeds within a remarkably limited range of age, and no significant difference was discernible for any of the breeds examined (fig. 25 and table 8). The same was true for the second and third incisors. Only the canine tooth differed slightly in that it tended to develop a little earlier in the beef breeds (fig. 25 and table 8).

Eruption into the Oral Cavity.—For the first three pairs of incisors, there was little distinction among the different breeds. Graph 2 and table 6 indicate that eruption of the first incisor in the Holstein-Friesian group was slightly in advance of the other

TABLE 8—(Continued)

		C. Comparison of eruption into oral cavity				D. Comparison of root completion			
Breeds	No. animals	First incisor (months)	Second incisor (months)	Third incisor (months)	Canine tooth (months)	First incisor (months)	Second incisor (months)	Third incisor (months)	Canine tooth (months)
Dairy and beef	869	23.0 ± 1	30.0 ± 1	35.7 ± 2	42.0 ± 2	28.0 ± 1	35.0 ± 2	43.5 ± 2	52.0
Dairy	495	23.0 ± 1	29.8 ± 1	36.0 ± 2	42.7 ± 2	28.2 ± 1	35.5 ± 2	42.6 ± 2	52.0
Holstein-Friesian	365	21.5	30.2	35.7	43.5	29.0	35.5	42.5	51.6
Guernsey	76	23.6	30.0	.....	42.0	28.2	.....	42.0	.....
Jersey*	33	.....	.....	35.4	.....	27.0	35.6	42.0	.....
Brown Swiss*	14	.....	.....	37.0	.....	28.0	.....	.....	.....
Ayrshire	7	.....	.....	.....	.....	.....	.....	.....	.....
Beef	374	23.0	30.6	35.3 ± 2	41.0 ± 2	28.0 ± 1	34.7 ± 2	44.4 ± 2	52.0
Hereford	258	23.1	31.0	34.8	41.0	28.5	34.3	45.5	52.5
Aberdeen Angus	100	24.0	30.0	36.0	.....	28.0	35.0	44.0	.....
Shorthorn*	16	.....	.....	.....	.....	28.0	.....	.....	.....
Bulls (all breeds)	105	.....	29.0 ± 1	35.5 ± 2	40.0 ± 2	28.4 ± 1	35.6 ±	42.0 ± 2	.....

* Extrapolated. ..... Inadequate material.

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breeds. In general, the canine teeth appeared to develop and to erupt a little earlier in the beef than in the dairy breeds (fig. 25 and table 8).

Completion of root formation.—Little difference was discernible for the first and second incisors and the canine teeth, but the third incisor appeared to complete its root formation a little earlier in the dairy animals (fig. 25 and table 8).

The bulls showed no consistent differences from the cows (table 8).

Conclusions.—The chronology of tooth development was found to be remarkably similar in all breeds of beef and dairy animals in spite of striking differences in other developmental processes and in genetic makeup. The chronology of tooth development in cattle appears, at first sight, to be a basic factor common to all breeds and does not appear to be linked to changing genetic factors or other developmental characteristics. However extensive experimentation would be necessary to prove this point.

Chronology of Premolar and Molar Development

It was thought that the chronology of tooth development would be incomplete without reference to the premolars and molars. Huidekoper[5] writing in 1891, tabulated the ages for the eruption of the anterior teeth, premolars and molars which had been worked out by different authors during the nineteenth century. Sisson, in 1953,[11] published a similar table of tooth eruption in cattle. None of these studies indicated the chronology of other phases of tooth development to cattle.

Methods.—Unfortunately, no simple technique was available for obtaining radiographs of the premolar and molar region in living animals. To overcome this lack, mandibles from animals of known ages were obtained from slaughterhouses. The mandibles were dissected free of other tissues and radiographs were obtained of the incisor and molar areas using our ordinary radiographic technique.

Because of the comparatively small number of specimens, it was necessary to extrapolate for the different stages of tooth development. The figures given (table 4) are therefore approximations.

Since many of the ages of the specimens were approximations, the physiologic age was determined by comparison with the stage of incisor development in the same jaw. This method of cross-dating proved satisfactory in that the stage of premolar and molar development was related to the stage of incisor development; and so permitted the determination of the sequence of tooth development (table 6).

Materials.—The dental material came from a slaughterhouse so the data was limited to 75 animals of indeterminable breeds and nutritional background, but of known ages which were equally distributed from birth to slightly more than two years.

Stages of Development

BIRTH

When the calf is born, the third deciduous premolar is usually erupted. It is followed soon by the second and first premolar so that by the second week, the deciduous dentition is complete except for the slow developing canine teeth. Interestingly, the order of development in the deciduous premolars (3rd, 2nd, 1st) is the reverse in the deciduous incisors (table 6).

At birth, the crowns of all the deciduous premolars (like the deciduous anterior teeth) are completely formed, while the roots are at different levels of development

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Legends for Figures 22-24 (Plate 4) on Opposite Page

Fig. 22—Radiograph of mandible of a 12-month-old Hereford steer. The first permanent molar is erupted and the second permanent molar has started to erupt, while the calcification of the third molar crown has just begun (A). The follicle of the second permanent premolar (B) is seen between the roots of its deciduous predecessor. Notice the large mandibular nerve canal (C).

Fig. 23—Radiograph of mandible of a 16-month-old Hereford cross-bred steer. The tooth germs of the three permanent premolar crowns can be seen lying between the roots of their predecessors. The second permanent molar crown is nearly completed.

Fig. 24—Radiograph of an adult Holstein-Friesian cow's mandible. The permanent dentition is complete.

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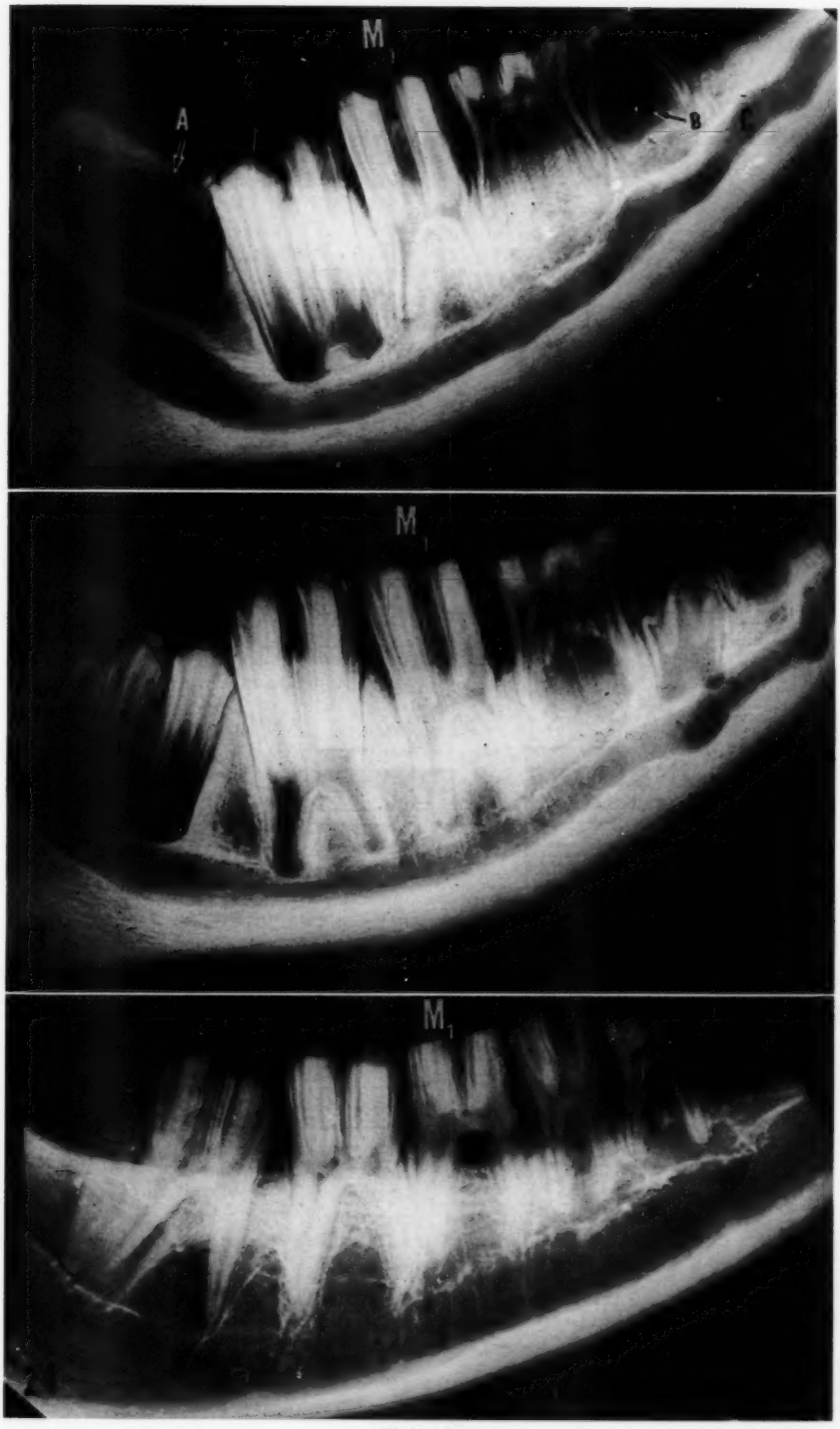

Plate 4

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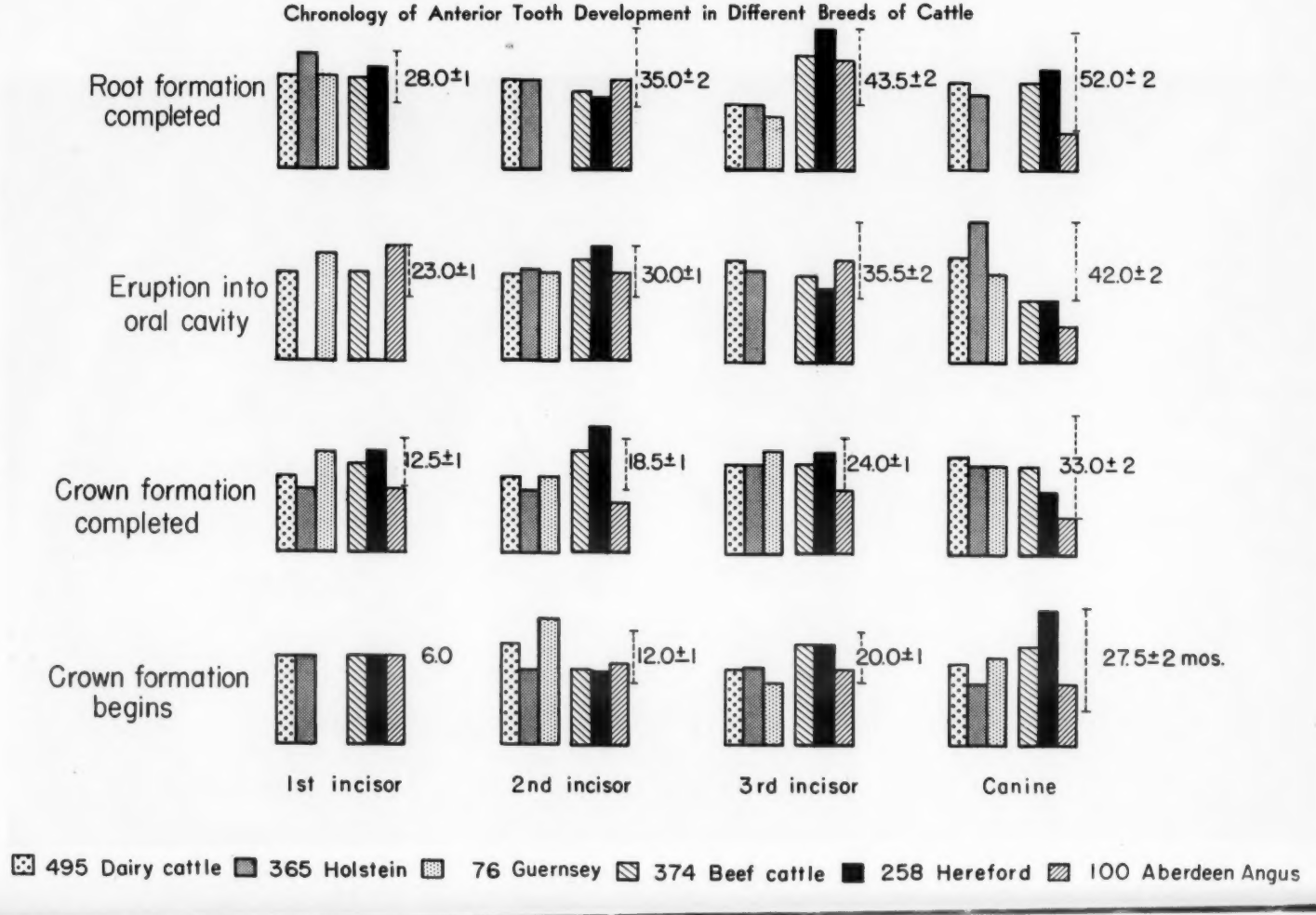
Chronology of Anterior Tooth Development in Different Breeds of Cattle
Root formation completed
28.0±1
35.0±2
43.5±2
52.0±2
Eruption into oral cavity
23.0±1
30.0±1
35.5±2
42.0±2
Crown formation completed
12.5±1
18.5±1
24.0±1
33.0±2
Crown formation begins
6.0
12.0±1
20.0±1
27.5±2 mos.
1st incisor
2nd incisor
3rd incisor
Canine
495 Dairy cattle
365 Holstein
76 Guernsey
374 Beef cattle
258 Hereford
100 Aberdeen Angus

(fig. 20). Root formation is completed approximately two months later (fig. 21).

Development of the first permanent molar teeth begins *in utero*. At birth, one third of the crown is calcified, and may be seen lying in its follicle posterior to the last deciduous premolar (fig. 20). Also visible is the mandibular canal which carries the nerve and blood vessels for the teeth and surrounding tissues. It runs from the middle of the ascending ramus, between the developing teeth and the lower mandibular border to the incisor region. It increases markedly in width with age (fig. 20 to 24).

Six Months

From birth until 6 months is the period of the deciduous dentition. With the eruption of the first permanent molar at 5 to 6 months of age, the mixed dentition period begins. This lasts until the deciduous canine teeth are exfoliated in the fourth year. The eruption of the first permanent molar into the oral cavity is coincident with the beginning of crown formation for the first permanent incisor (fig. 9).

The first permanent molar is the smallest of the permanent molar series, while the second and third are the larger.

Since the first permanent molar begins to calcify before the animal is born and does not complete its crown until the third month of life, the neonatal line marking the trauma of birth should be found within the enamel and dentin which was forming and calcifying at the time of birth.

Twelve Months

The second permanent molar may be partly erupted at 12 months of age (fig. 22). This molar begins to calcify soon after birth (fig. 21) and it takes approximately a year for the crown to form (table 4). This is twice as long as required for the formation of a permanent incisor crown.

Intra-osseous eruption of the molars begins soon after the crown is completed. This is much earlier than was observed in the incisors, which do not begin to erupt until their roots are one third completed.

Formation and calcification of the third molar, and the three premolars begin at approximately the same time, between 9 to 13 months of age (fig. 23). The crowns are completed approximately one year later (table 4). Emergence into the oral cavity follows soon after the crowns are completed, between the second and third year of life, the order being second premolar, third molar, first premolar, and third premolar. The molars erupt into the oral cavity soon after intra-osseous movement begins because, unlike the anterior teeth, the crowns of these teeth develop close to the surface of the jaws. Intra-osseous eruption begins early (when the crown is completed) and terminates quickly because little movement is necessary to bring these teeth into function. The incisors and canine teeth, when they have erupted, lie high above the basal bone, almost entirely within the alveolar process, while the molars, when they first erupt, are largely within the basal bone and have only a small alveolar process. However, as the molars continue to erupt slowly during function, they migrate away from the lower border of the mandible and the basal bone. At the same time, bone forms at the alveolar crest to give additional support to the erupting tooth, and thus the height of the alveolar process increases throughout life (fig. 20 and 24).

Two Years

The second and first premolar teeth erupt into the oral cavity at 24 to 30 months, between the appearance of the first and second permanent incisors followed soon after by the third and first premolars (table 5). The second year is, therefore, characterized by a rapid displacement of many of the deciduous incisors and premolars by their permanent successors.

Three Years

By the third year of life, all the premolars and molars are erupted and three of

Legend for Figure 25 on Opposite Page

Fig. 25—Composite graphs comparing the chronology of tooth development in different breeds of dairy and beef cattle (± indicates 1.5 standard deviations around the mean and is indicated by the vertical dotted line).

the four permanent anterior teeth are also erupted (fig. 24). The permanent dentition is almost complete, except for the canine teeth. The last tooth emerges after an interval of approximately six months (3½ years) to complete the permanent dentition. This delay supports the thesis that the fourth anterior tooth is related to the late developing canine tooth of other species.

Discussion

Genetic Differences

Little difference was observed in the chronology of any phase of tooth development, including eruption, between the various breeds (fig. 25). When a marked difference did occur, it was usually associated with a shortage of data. This suggests that the chronology of tooth development may not be related nor influenced by genetics in the same way that tooth or jaw morphology are. It suggests that the differences in eruption age observed in man may be better related to environment factors such as diet, occlusion, size of jaws, etc., rather than to genetic differences. However, these considerations can only be confirmed by planned experimentation.

Sex Differences

The bulls were not consistently more precocious nor more retarded in any one of the tooth developmental processes considered.

The fact that all the animals were on optimal levels of nutrition may well be a contributing factor to the small difference of tooth chronology found.

Perhaps the chronological pattern may be a guide to nutritional status. The analysis of chronology of tooth development of a sample of continuously undernourished animals would be a worth while study.

Order of Eruption

The six-month (first permanent) molar is the bovine equivalent of the human 6-year-old molar, while the 12-month second permanent molar is equivalent to the human 12 year (second permanent) molar.

The third permanent molar and the three premolars erupt as a group, just as the premolars and second molars do in man.

A fixed and definite order in the eruption of this premolar and molar group (P2-M3-P1-P3) is implied (table 7). However, this order may well be changed in a larger or wider sample under different local conditions, diet and occlusion, as it does in man.

The "Aging" of Animals

From time immemorial, the age of many species of domestic animals has been assessed by the state of eruption and wear of their teeth. This study suggests the possibility of using intra-oral radiographs for assessing the age of animals more accurately during the first four years of their life. The accuracy of this method is indicated by the narrow range in which the developmental process takes place.

A controlled, double-blind study comparing estimations of age from clinical observations of teeth with estimations made from radiographs in a group of animals with accurate birth records would be most revealing.

Tooth Ring Analysis

As indicated before, the enamel- and dentin-forming cells are extremely sensitive to systemic changes in their environment while they are actively engaged in the deposition and calcification of the enamel and dentin matrix. Any metabolic change in the organism affects the quality and sometimes the quantity of matrix deposited by these cells. These are seen as accentuated incremental lines in the enamel and dentin and, if the disturbance is severe enough, hypoplastic defects result.[12]

In human teeth, examination of the rings or layers of enamel and dentin formed during different periods of development show that the prenatal enamel is much better and homogeneously calcified than the postnatal enamel, the two portions being separated by a prominent incremental line or ring at the exact level of enamel and dentin forming at birth. This neonatal ring is a valuable landmark which aids in the assessment of prenatal as well as postnatal injuries or defects in development. Massler, Schour, and Poncher [12] also describe differences in the degree of calcification of enamel formed during infancy, early childhood and later childhood. They found accentuated lines occurring con-

stantly at the level formed at 10 months of age (infancy ring), 2½ years (early childhood ring), and at 4½ years (later childhood ring). They suggest that such metabolic changes as weaning and puberty should leave permanent records in the structure of the enamel and dentin.

The teeth of cattle offer a valuable opportunity to study the effects of other metabolic episodes on the form and calcification of enamel and dentin. It is certain that there is a neonatal line in the first permanent molar and, since cattle are bred while enamel and dentin are still forming, the effects of pregnancy should, therefore, be registered in the enamel and dentin forming at 18 to 24 months (cervical area of 1_2 and 1_3). Clearly this hypothesis may be tested by comparison of the teeth of cows with the teeth of bulls or steers.

Preliminary observations in a number of ground sections of teeth indicate that the enamel and dentin of the incisors contain a relatively large number of prominent incremental lines; also, that this rapidly growing structure is extremely sensitive and surely records equivalent metabolic periods observed in the slower growing human enamel. Furthermore this enamel may register the effects of nutritional variations (summer and winter feed) in its enamel as well as minor infections and systemic ailments.

With the chronology of tooth development in cattle better established, it will be possible to make a detailed analysis of the different incremental lines that have been noticed. By examining the teeth of animals with precisely recorded developmental and nutritional histories, these incremental lines may be correlated to different physiological stresses as weaning, pregnancy, and lactation or different nutritional experience. Furthermore, the severity of mottling as caused by ingestion of excessive amounts of fluorides can be related to the animal's physiologic development.

Summary and Conclusions

This study was designed to establish the chronology of enamel and dentin formation in cattle as accurately as possible in order to lay a firm foundation for future studies in tooth ring analysis, *i.e.*, the effects of specific physiologic and pathologic stresses on enamel and dentin formation.

The chronology of development of the permanent incisor and canine teeth was established from analysis of radiographs obtained *in vivo* from 869 purebred cattle living under optimal nutritional conditions. It was found that development of the incisor and canine (anterior) teeth followed an orderly sequence. The first incisor began to develop enamel and dentin when the calf was 6 months of age; the second incisor at 12 months, when the crown of the first incisor was completed; and the third incisor at 20 months, soon after the crown of the second incisor was completed at 18 months. The canine tooth began its enamel and dentin formation at 27 months, three months after the third incisor crown was completed.

Eruption and root formation followed a similar orderly sequence from the first incisor to the canine tooth. The first incisor emerged into the oral cavity at 23 months, the second incisor at 30 months, the third incisor at 36 months and the canine teeth at 42 months. Root formation was four fifths completed at the time of emergence.

No significant differences in the chronology of tooth development in the different breeds or sexes was found in spite of relatively large developmental and genetic differences between dairy and beef cattle.

It is hoped that this study will provide a satisfactory baseline for future studies which relate the effects of physiologic stress such as birth, weaning, pregnancy, and lactation as well as pathologic factors as nutritional deficiencies, fluorosis intoxication, and infectious diseases upon the enamel and dentin (tooth ring analysis).

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A Revised Concept of the Pathologic Changes of the Tongue in Cattle with Vesicular Stomatitis

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VESICULAR STOMATITIS in cattle is classified as a viral disease characterized chiefly by the formation of vesicles, or blisters, in the mouth.[2-6, 8-10, 12-15, 17] Lesions on the teats and feet have also been reported, but the following discussion will be limited to consideration of lesions of the tongue.

Published descriptions of the gross pathology are similar. The concept of the morphogenesis of the oral lesions is as follows: They begin as small reddened patches[10, 13, 15] or as raised, flattened, pale pink to blanched papules[2, 14] that may be difficult to detect even on close observation.[2] They enlarge and develop rapidly into vesicles "as small as a dime or as large as a silver dollar"[3] (1.5 to 4.5 cm.). The vesicles are only slightly elevated and are filled with clear or yellowish serous fluid.[10] Adjoining vesicles may coalesce, or new vesicles may form around original ones and then coalesce, so that large areas of the mucosa become affected.[13-15] The vesicles usually rupture in a short time.[2, 3, 9, 13, 15] Their epithelial covering may remain attached at their borders for a time, but is soon broken away, exposing the raw underlying surfaces.[3] The lesions then appear as reddened erosions with grayish white fragments of the torn mucous membrane remaining attached to their irregular borders like a fringe.[3, 6, 9, 13, 15] The epithelial tissue regenerates quickly and healing is complete in one or two weeks provided secondary bacterial invasion of the denuded areas does not take place.[9, 14] In the latter event, the nature of the superinfected lesion will be changed and the healing period prolonged.[2]

Thus the oral lesions are considered to be basically vesicular in character. It is believed that all the lesions pass through a transient stage of gross vesiculation that may or may not be observed clinically. Gross vesiculation is commonly called a blister, and blister is defined as a "collection of serous fluid causing a bladder-like elevation of the cuticle" (Webster), or "a localized collection of fluid in the epidermis causing an elevation of the stratum corneum and its separation from the underlying parts" (Dorland). Lesions which have the epithelium *in situ* and do not show a definite, grossly visible, fluid-filled cavity are considered to be either immature or early vesicles, or vesicles that have lost their fluid and have collapsed without sloughing their epithelial covering.

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Observations by practicing veterinarians[1, 11] of vesicular stomatitis in cattle indicate that the finding of grossly evident vesicles in the oral mucosa of naturally infected cattle can be more unusual than routine. In discussing the outbreak reported by Brandly *et al.*,[1] McDermid stated, "The early lesions consisted of small, brownish, slightly raised, rounded areas, which were frequently multiple. No vesicles were present. The thickening consisted of an increase of the surface epithelium which was readily removed by scraping. The lesions, being generally multiple, soon extended along the mucosa until the larger portion of the dorsum, upper lip, and gum were involved . . . Of course, upon examination, one found various degrees of ulcerative and necrotic erosion in the mouth and on the tongue. . . . We did not find any vesicles or blisters in the mouths of either cattle or horses."

In commenting on the same outbreak, Brandly *et al.*[1] stated, "The almost complete failure to observe (gross) vesiculations among the field cases led to some doubts among practicing and other veterinarians as to the identity of the malady even after isolation of the virus and its identification as that of vesicular stomatitis. Examination . . . of a number of animals in each of 11 herds on a single day failed to demonstrate a single vesicle. However, in a few animals there were areas of freshly detached mucous membrane which had evidently constituted the surface of a vesicle recently ruptured." The general character of the lesions was described as "extensive maceration and exfoliation of the (oral) mucous membrane."

In another natural occurrence of the disease among cattle in the Little River area of Oklahoma and Arkansas, actual gross vesicles were seen in only 3 of 1,000 cattle affected.[11]

The low incidence of typical gross vesicles observed in farm and ranch animals with natural infection has been attributed to rapid development, fragility, and early rupture of the vesicles.[1, 2, 9, 10, 12] Instances have also been reported[1] where lesions in a stage of gross vesiculation were not seen in experimentally infected cattle under close observation. The difficulty of finding vesicles in cattle involved in natural outbreaks of vesicular stomatitis may cause a delay in the recognition and diagnosis. An early diagnosis of all vesicular conditions is important in view of the ever-present threat that foot-and-mouth disease might gain entrance into the country and become established. The possibility has been recognized[10] that, under certain conditions, oral lesions of foot-and-mouth disease in cattle

may also show a low percentage of gross vesiculation.

The purpose of this paper is to report observations on the development and behavior of the lingual lesions of vesicular stomatitis in cattle, with emphasis on the occurrence of grossly evident vesicles during the course of the disease. Since the diagnostician in the field is concerned with the over-all pathologic picture of the vesicular diseases as well as the sometimes less common "typical lesion," effort has been directed toward study of the former. The common practice of selecting a few "typical" or "good" lesions for study was abandoned, since that would serve only to confirm the already established academic concept of the pathologic process and would not elucidate the total pathologic picture. A special technique was designed to furnish sufficient lesion material for study of the total lingual pathology and to make possible detailed gross, subgross, and microscopic correlation of the lingual lesions of vesicular stomatitis. The subgross examination was made in a visual range between gross and microscopic, using enlarged pictures (photomacrographs) of cross sections of the lesions. The data on vesiculation thus obtained provided a new concept of the nature of the pathologic changes of the bovine tongue in vesicular stomatitis.

MATERIALS AND METHODS

Twenty Hereford steers, 18 to 24 months old, inoculated intradermolingually with vesicular stomatitis virus, were used in this study. Ten were inoculated with the fourth bovine passage of New Jersey-type virus, Concan strain (isolated in 1949 from a cow at Concan, Texas). The titer of the inoculum was $10^{6.78}$ $i.d._{50}$/ml. The other 10 animals were inoculated with the ninth bovine passage of Indiana-type virus (Fort Lupton strain isolated in 1942 from a horse at Fort Lupton, Colo.). Prior to passage in cattle, this virus had undergone 36 passages in horses. The titer of the inoculum was $10^{5.82}$ $i.d._{50}$/ml. Both strains of virus were obtained from the Animal Disease Station of the Agricultural Research Service at Beltsville.

The inoculations were made for viral titration purposes, using four dilutions of virus and injecting 0.1 ml. of each dilution into five sites on the tongue (fig. 1) in the manner described by Henderson [7] for titration of foot-and-mouth disease virus. The original undiluted inoculum was a 10 per cent suspension of epithelium in tryptose phosphate broth, pH 7.4, to which was added 1,000 units of penicillin and 1,000 mg. of dihydrostreptomycin sulfate per milliliter. New Jersey-type virus was inoculated in 10^3 to 10^6 dilutions, inclusively, and Indiana-type virus in 10^2 to 10^5 dilutions, inclusively.

Clinical observations of the tongues were omitted in order to avoid interference with natural development of the lesions. Four of the 10 steers inoculated with each type of virus were killed on the second day (47 to 49 hr.), 4 on the third day (71 to 73 hr.), and 2 on the fourth day (95 to 97 hr.) following inoculation. Necropsy was performed immediately after death.

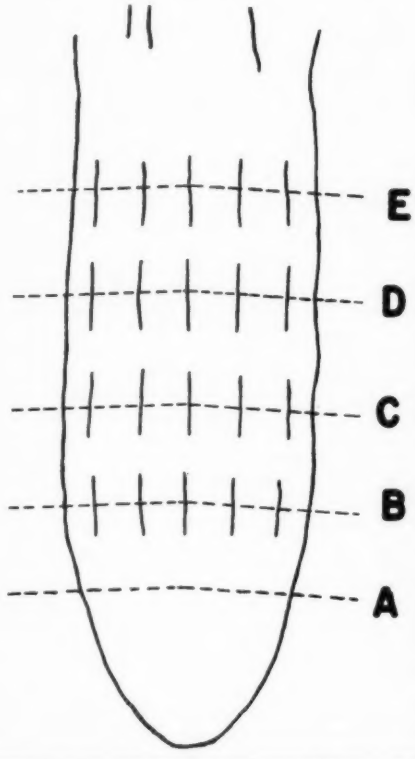

Fig. 1—Diagram of bovine tongue showing sites of titration inoculations (vertical lines) and areas where tissue sections for histopathologic examination were taken (broken lines). Lowest dilution of virus was inoculated at E, highest dilution at B. A represents uninoculated (control) area.

Special Technique.—The tongue of each steer was removed at necropsy, washed gently to avoid damage of the lesions, and photographed on 4- by 5-in. Kodak Tri-X film. The entire tongue was then hardened 24 hours or more in 15 per cent formalin solution and cut in half longitudinally; ten tissue blocks for histological sections were then taken from places shown by dotted lines in figure 1. The eight large blocks taken at B, C, D, and E provided a composite picture of all 20

inoculation areas, and the two blocks taken at A provided a picture of an uninoculated (control) area of mucosa at the tip (fig. 1). The blocks were large, but in every instance fit between the knife clamps of a Spencer Rotary Microtome, and were no more difficult to section than had they been subdivided.

The tissue blocks were returned to 15 per cent formalin solution to complete fixation. Identification was maintained by placing each pair of blocks in a different bottle and trimming the muscle closer in blocks from the right side of the tongue than in those from the left side (fig. 5b). When fixation was complete, the tissue blocks were embedded in paraffin, cut in sections 7 μ thick, and stained with hematoxylin and eosin. The embedding was done with the tissue blocks face down (in reverse position) so that the stained sections could later be arranged in proper order in relation to this location on the tongue.

After the coverslips had set, the sections from each tongue were arranged in proper order and photographed with strong diffuse transillumination on 4- by 5-in. Kodak Tri-X film. Prints of the tongues and of the tongue sections were made with an enlarger on 8- by 10-in. Kodabromide No. 2 paper. The pictures of the sections were larger than natural size and were, therefore, designated "photomacrographs."

Pictures of tongues are illustrated in reduced size (fig. 5a–8a, 12a, 14a). Photomicrographs of tongue sections for subgross examination are illustrated reduced to approximately natural size (fig. 5b–8b, 12b, 14b). Lesions in the mucosa can readily be distinguished from normal mucosa in the photomacrographs by comparing sections A, taken from the unaffected tip of the tongue, with sections B, C, D, and E. Grossly evident vesicles can also be identified as areas of mucosa that have separated from the underlying tissue, enclosing either a spacious or a slitlike cavity between the two. The 8- by 10-in. prints used for study in the laboratory are much easier to read than the reduced illustrations.

PATHOLOGY

The character of gross lesions is determined by their histopathologic features. Therefore, in order to analyze the pathologic topography of the infected tongues it was necessary first to study the histopathologic alterations and then to work out correlations between the histopathologic, the subgross pathologic (as seen in photomacrographs of the tongue sections), and the gross pathologic changes (as seen in tongue pictures). The term lesion will be used to designate (1) circumscribed areas of pathologic reaction to single inoculations and (2) coalescent pathologic reactions resulting from two or more closely spaced inoculations.

Histopathology.—The basic histopathologic alterations of lesions in the lingual mucosa were (1) intercellular edema, (2) necrosis of epithelial cells, and (3) inflammatory cellular infiltration. A combination of these histopathologic alterations was the basis of all gross lesions on the tongues whether or not gross vesiculation developed. This held true in lesions caused by either Indiana- or New Jersey-type virus.

Intercellular Edema.—The intercellular edema occurred in the middle of the malpighian layer. A narrow zone of epithelial tissue adjacent to the basal layer usually remained relatively unaffected. Often it was the only alteration seen at the lateral edges of a lesion (fig. 2). The epithelial cells became separated by vacuolar and larger intercommunicating cavities originating in the intercellular spaces. The intercellular bridges were disrupted wherever separation took place. The cells became stretched and misshapen by pressure of the fluid but showed no other morphologic changes that could be attributed to the edema. Even in the most severely edematous areas, the epithelial cells did not become separated completely from each other but remained attached sufficiently to effect a lacelike histologic picture (fig. 2).

Necrosis of Epithelial Cells.—Necrosis of epithelial cells was found in all lesions except, in some instances, at the lateral edges, where intercellular edema alone was observed. In places where the basic pathologic alterations had not fully developed, there was less edema and more cells were undergoing necrosis in the lower portion of the malpighian layer than in the upper portion (fig. 3). This was an indication that epithelial necrosis was concomitant with, but not consequent to, the intercellular edema. However, the fact that severe edema without appreciable necrosis was seen at the edges of lesions indicates that the intercellular edema occurred somewhat earlier than necrosis in development of the initial lesion.

The cytoplasm of epithelial cells undergoing necrosis was more strongly eosinophilic than that of normal cells. The nucleus was pyknotic to a variable degree. As the nucleus contracted, the basophilic chromatin either condensed into irregular, dark-staining clumps at the periphery or dispersed uniformly throughout the nuclear

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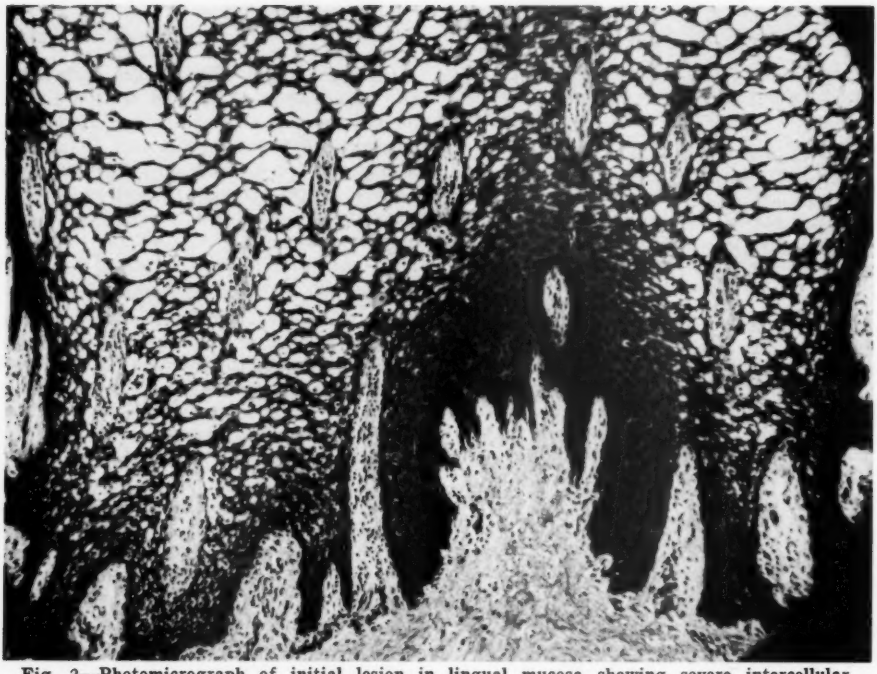

Fig. 2—Photomicrograph of initial lesion in lingual mucosa showing severe intercellular edema, but no appreciable epithelial necrosis or cellular infiltration. x 80.

Fig. 3—Photomicrograph of initial lesion in lingual mucosa showing epithelial necrosis near basal layer, also some intercellular edema and cellular infiltration. x 185.

sap, giving such altered nuclei a uniform, slate-gray coloration in sections stained with hematoxylin. The eosinophilic alteration of the cytoplasm developed more rapidly than the changes in the nucleus. Eventually, most of the epithelium in the lesions became necrotic except for some cells at the base of the conical papillae and occasional patches of basal epithelium between the conical papillae. There also were some isolated epithelial cells and patches of epithelium within the malpighian layer that appeared to remain unaffected by the virus. These cells were not in a location where their survival would affect the character or course of the lesions. The epithelium of all the fungiform papillae seen in lesion areas appeared normal and showed no morphological evidence of any effect from the virus.

There was no evidence that the necrosis was lytic in character. Some necrotizing cells may have been shrunken but, in most instances, they maintained size and shape comparable to neighboring cells that were not yet undergoing necrosis (fig. 3). Subsequent dissolution of necrotic epithelial cells was frequently observed, but only in areas of well-marked cellular infiltration where digestion by proteolytic enzymes of leukocytes and phagocytosis by monocytes could be held responsible. As the initial lesions developed, dissolution of necrotic epithelium became evident in locations close to and sometimes including the basal layer. An initial lesion in a relatively mature stage of development, prior to either vesiculation or erosion, is shown (fig. 4).

Inflammatory Cellular Infiltration.—The cellular infiltrate consisted essentially of heterophil granulocytes and medium-sized mononuclear cells, classified as monocytes. Cellular infiltration appeared to develop in proportion to, and in relation with, epithelial necrosis. The number of monocytes in the infiltrate was surprisingly large. Initial lesions approaching a mature stage of development showed infiltrating cells dispersed throughout the edematous, partially necrotic, upper portion of the mucosa and concentrated in the more completely necrotic lower portion of the mucosa near the basal layer (fig. 4). In some places, the concentrations of cellular exudate attained the proportions of microabscesses. Inflammatory cellular infiltration of the lamina propria was mild in degree and consisted mainly of heterophil leukocytes and

Fig. 4—Photomicrograph of mature initial lesion (without gross vesiculation) in lingual mucosa. There is marked intercellular edema in the middle and upper portion of the malpighian layer. Most of the epithelium is necrotic, except that covering the base of the conical papillae and some of the basal layer between the conical papillae. Cellular infiltration can be seen in all areas, except the stratum corneum. x 27.5.

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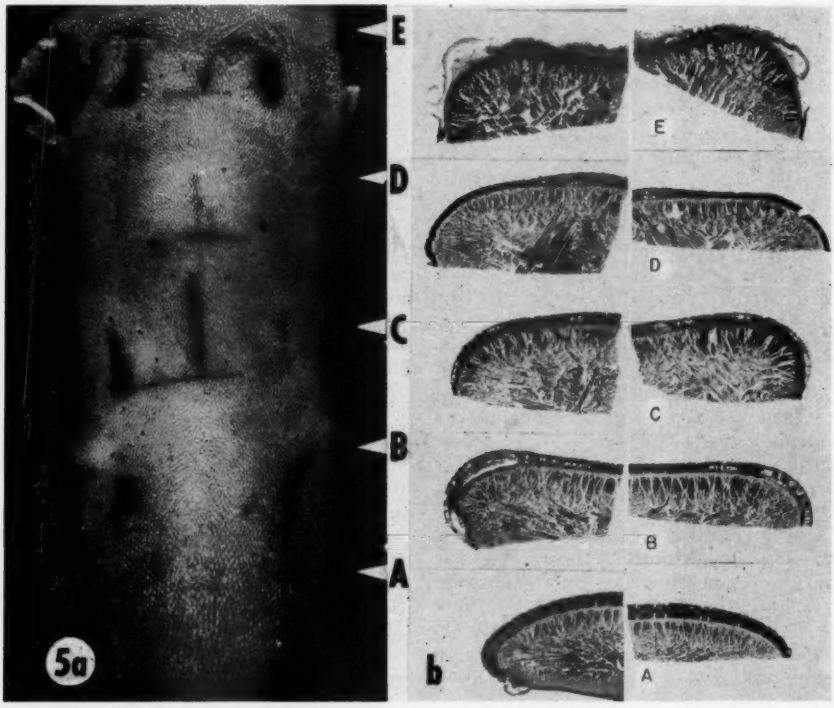

Fig. 5a—Photograph of bovine tongue two days postinoculation. Gross vesicles are present at B, left border, and at E, left border and right border. The blanched, irregularly elevated or swollen mucosa in other inoculation areas represents initial, nonvesiculated lesions that have coalesced. Notice beginning erosion of nonvesiculated lesion at E, dorsum. The dark vertical patches are areas of hemorrhage from trauma at some sites of inoculation.

Fig. 5b—Photomacrograph of sections from tongue shown in figure 5a. Gross vesicles are shown at B, left border, and at E, left border and right border. A small vesicle is present on the underside of the tongue at A, left border. The entire lesion at A is not a secondary lesion but an extension of lesion from B. Nonvesiculated lesion area is either lighter or less dense than the normal mucosa, which is shown at A, dorsum and right border, at B, center dorsum, and at D, left and right sides and borders.

monocytes in transit to the mucosa. The amount of cellular infiltration was increased in areas where gross vesiculation had occurred.

Subgross Pathology.—After the basic histopathologic changes had been determined, photomacrographs of the lesions were examined to study the process of gross vesiculation and to determine the amount and the role of gross vesiculation in their development. The term "gross vesiculation," as used in this discussion, is defined as "development of fluid-filled cavities in or beneath the lingual mucosa that are large enough and definite enough to be recognized on clinical examination." This type of lesion, or stage of lesion development (the grossly apparent vesicle), is of utmost clinical importance. The malady is diagnosed clinically as one of the vesicular diseases on the basis of gross vesicle formation. Actually, the term "vesicle" is used loosely in connection with the vesicular diseases of animals. It refers to fluid-filled cavities (blisters) of all sizes. The term "bulla" would apply more correctly to the larger "vesicles," but has not been used extensively in veterinary practice. Obviously, a study of vesiculation would not be complete without observations on the development and final outcome of initial lesions that failed to undergo gross vesiculation.

Photomacrographs of the tongue sections were studied and the subgross appearance

of the diseased mucosa was correlated with its histologic appearance under low-power magnification. Photomacrographs of representative tongue sections (fig. 5b, 6b, 7b, 8b, 12b, and 14b) are arranged the same as illustrated in figure 1. The two sections taken at A through the uninoculated tip of the tongue served as controls and are located at the bottom of the picture. The four pairs of sections taken at B, C, D, and E show lesions that developed from the titration inoculations. The smallest dose of virus was inoculated at B and the largest at E.

Two Days Postinoculation.—Lesion development at 48 hours postinoculation is shown (fig. 5b, 6b, 7b, and 8b). Normal mucosa at A from the uninoculated tip of the tongue is darker and more opaque than lesions at B, C, D, and E. Only in one instance (fig. 5b) did a pathologic reaction occur in the control region (A, left side), probably as a result of extension from the adjoining region (B). A large amount of initial lesion that has not undergone gross vesiculation is shown (fig. 7b). In subgross appearance, areas of initial lesion are irregularly light and clear as compared with normal mucosa, on account of the intercellular edema and epithelial necrosis.

Classical gross vesiculation including both ruptured and unruptured vesicles can be seen (fig. 5b—B, left, and E, left and right; and fig. 6b—B, right, D, right, and

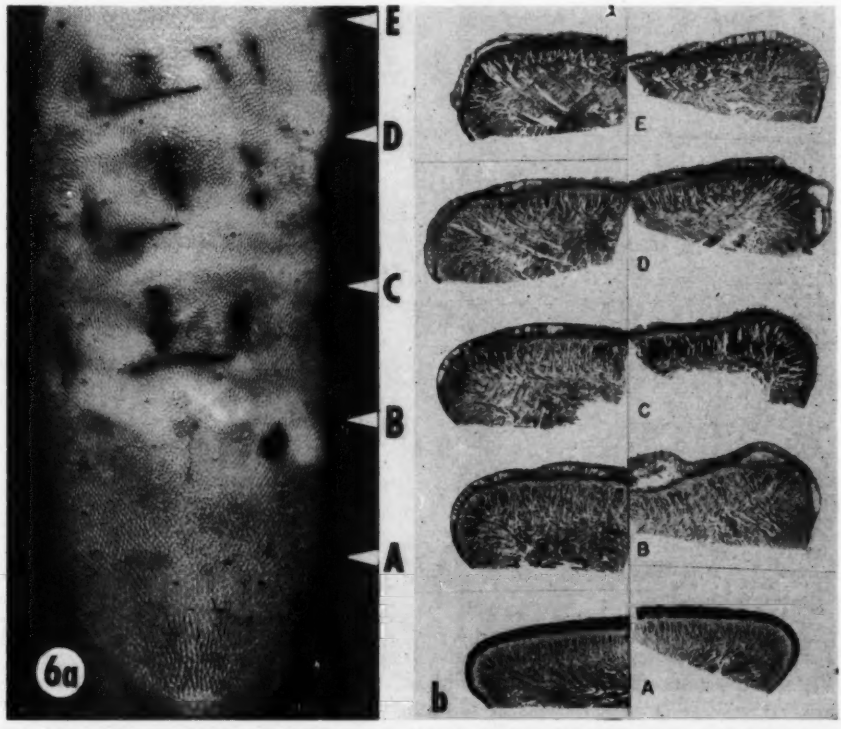

Fig. 6a—Photograph of bovine tongue two days postinoculation. Gross vesicles are seen at B, dorsum and right border, and at D, right border. The blanched, irregularly elevated or swollen mucosa in other inoculation areas represents initial, nonvesiculated lesions that have coalesced. The dark vertical patches are areas of hemorrhage from trauma at some sites of inoculation.

Fig. 6b—Photomacrograph of sections from tongue shown in figure 6a. Gross vesicles are present at B, dorsum and right dorsum, at D, right border, and at E, left border. The rest of the mucosa shows either the light or the clear character of nonvesiculated initial lesion except the normal mucosa at A, a small patch at B, left dorsum, and a small patch at D, left dorsum.

E, left). Subgross histological correlation showed that the gross vesicles developed in areas of initial lesion simply by a breaking loose or separation of the necrotic, edematous mucosa from the underlying tissue, forming a cavity filled with predominantly fluid exudate. The line of separation was in the region of the basal layer. Portions of the rete pegs that had not undergone necrosis remained attached to the floor of the vesicle, along with papillae of the lamina propria that had been pulled loose from the diseased mucosa. A low-power photomicrograph of the edge of a classical vesicle is shown (fig. 9).

Less conspicuous gross vesiculation is shown (fig. 8b—C, left, D, left, and E, right). These vesicles have collapsed almost completely and appear as slitlike spaces beneath the diseased mucosa at the lateral borders of the tongue. Fluid had evidently escaped but, from their contour, it appears that the vesicles never had become as fully distended as those shown in figures 5b and 6b. Loose flaps of detached pathologic mucosa at each border of the tongue (fig. 7b—B, left and right) evidently represent gross vesicles that had ruptured.

Considerable study was made of initial lesions, which had not undergone gross vesiculation. All these lesions appeared lighter than normal mucosa in the photomacrographs, but showed considerable variation in density. The range in density

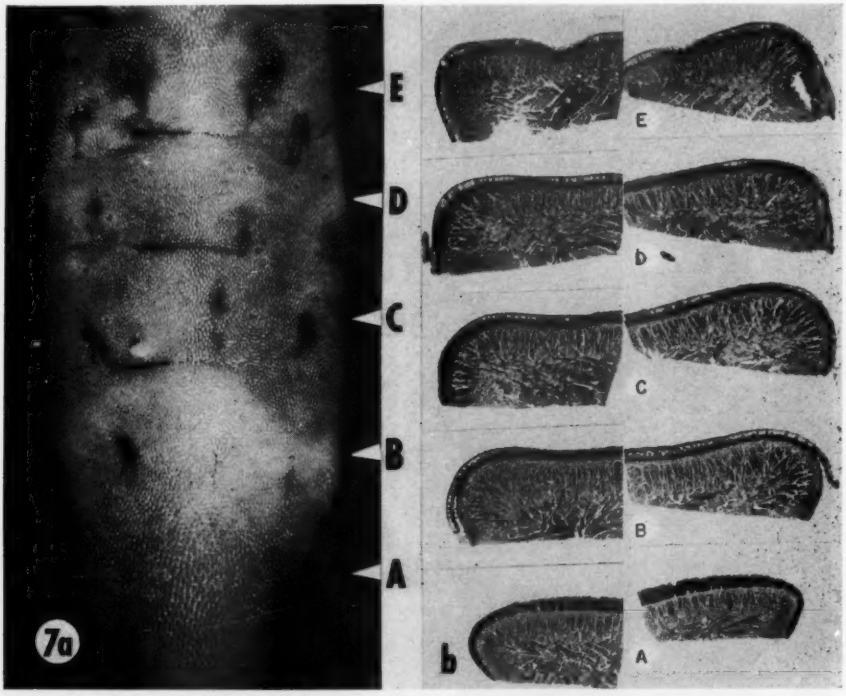

Fig. 7a—Photograph of bovine tongue two days postinoculation. Possible gross vesiculation is difficult to evaluate and most of the mucosa in the areas of inoculation shows only the characteristic swelling (slight elevation) and blanching of initial lesions. Notice the rough areas of beginning erosion at E, right and left dorsum. The dark vertical patches elsewhere represent areas of hemorrhage from trauma at some sites of inoculation.

Fig. 7b—Photomacrograph of sections from tongue shown in figure 7a. Loose flaps of mucosa representing ruptured vesicles are seen at B, left and right borders. Deep erosions, presumably representing previous vesicles are shown at D, right border, and at E, right border. The remainder of the mucosa in the inoculation areas is in a stage of nonvesiculated initial lesion. The clear areas are in the necrotic-edematous stage; the dense areas are undergoing dehydration. Notice the beginning erosion at E, right and left dorsum. Normal mucosa is shown at A and at B, left dorsum.

varied from practically clear to a dense gray that could just be distinguished from the darker gray of normal mucosa.

Subgross histologic correlation showed that variations in density of nonvesiculated lesions depended on variations in the amount of intercellular edema. The clear, light-colored lesion areas were the most edematous; the denser, darker ones the least edematous. Some of the denser, darker lesion areas showed little intercellular edema. An explanation of this variation became evident upon further detailed subgross histologic correlation. The lesion which was most edematous had an intact, unbroken stratum corneum, seen as a thin, dark line in the photomacrographs. The denser, darker, and less edematous lesion was somewhat rough or ragged on the surface as shown in the photomacrographs.

Microscopic examination showed cracking and beginning erosion of the stratum corneum (fig. 10). Vertical cracks were seen developing adjacent to the conical papillae (fig. 11). It thus appeared that the dense lesion areas were simply initial (necrotic, edematous) lesions from which the fluid component had seeped through cracks at the surface (fig. 10, 11). This supposition was further confirmed by the fact that all dry areas contained considerable amounts of cellular exudate trapped among the necrotic epithelial cells in the upper portion of the mucosa. The amount of cellular exudate in the dry areas could not have accumulated there without the aid of considerable fluid, the pre-existing intercellular edema.

Tongues with a predominance of nonvesicular lesions in the initial necrotic, edematous stage and with a predominance of nonvesicular lesions in the drying stage are shown (fig. 7b and 8b, respectively). Both stages of nonvesicular lesion were seen two days postinoculation in all tongues infected with either the New Jersey- or the Indiana-type of vesicular stomatitis virus.

At this point, it was evident that the pathogenic effect of vesicular stomatitis virus on the lingual mucosa was the production of initial lesions characterized by intercellular edema, epithelial necrosis, and inflammatory cellular infiltration. In 48 hours these initial (necrotic-edematous) lesions might undergo gross vesiculation by the process of separating from the underlying tissue, or might begin to dry *in situ* by seepage of edema fluid through the damaged surface.

The areas of vesiculation and the areas of nonvesicular lesions shown in the photomacrographs of the eight tongues examined two days postinoculation were measured in millimeters and the percentage of gross vesiculation was calculated from these figures (table 1). The measurements were made on the basis of subjective interpretation of what is and what is not a gross vesicle. Since small areas of mucosal separation that could well be overlooked on clinical examination were classified as vesicles, the measurements made were weighted in favor of vesiculation. Since just linear, rather than two dimensional, measurement of the lesions was possible, the percentage figures serve only as a practical estimate of gross vesiculation that occurred. This was less than 30 per cent of the total lingual lesion caused by Indiana and New Jersey types of vesicular stomatitis virus.

TABLE 1—Percentage of Gross Vesiculation in Titration Areas

Titration area	2 days postinoculation		3 days postinoculation	
	Virus type		Virus type	
	Ind. (%)	N. J. (%)	Ind. (%)	N. J. (%)
Area E	18*	51	12	20
Area D	20	28	9	22
Area C	11	9	12	0
Area B	28	19	6	8
All areas	19	28	10†	15†

* Each figure represents measurements of lesions on 4 tongues.

† The lesser percentage of vesiculation three days postinoculation compared with two days postinoculation is not considered significant, except to show that gross vesiculation did not continue to develop after the second day postinoculation.

As judged by examination of the photomacrographs, none of the tongues were completely devoid of vesiculation but, in some (fig. 7b, 8b), collapse of the vesicles had already occurred without subsequent development of characteristic raw, red erosions. Such tongues would be poor material for clinical diagnosis of a vesicular disease, despite the large amount of lesion area indueed by 20 separate inoculations of virus.

Most of the vesicles developed along the lateral borders of the tongue and in the dorsum at B near the tip. Friction of the lateral borders of the tongue with the teeth,

and of the dorsum at B with the dental pad probably contributed to the formation of the vesicles. Furthermore, the mucosa at the lateral borders is not so thick or so firmly anchored to the submucosa by the conical papillae as the mucosa of the dorsum.

Two of the eight tongues examined two days postinoculation showed considerable patchy separation of the pathological epithelium of the dorsum at E, where the highest dose of virus was injected (fig. 5b). This was classified as vesiculation, yet on clinical examination the affected mucosa would appear to be sloughing. This indicates that severity of exposure to the infection is also a factor in determining the character of the lesions. It also was of interest that the combination of intercellular edema and necrosis was necessary for development of gross vesicles in the lingual mucosa. Severely edematous areas of mucosa (fig. 6b—B, left, lateral border, and C, left, lateral border), with a minimal amount of necrosis, had not undergone gross vesiculation.

Three Days Postinoculation.—Apparent gross vesiculation was minimal at three days postinoculation regardless of the type of virus inoculated. Vesicles present on the second day had already ruptured, and their covering had been lost. However, the gross vesiculation was estimated by measuring total lesion area and areas of deep erosion where vesicles apparently had been present, and the percentage of gross vesiculation was calculated for each type of virus (table 1). Despite the inherent inaccuracy of the

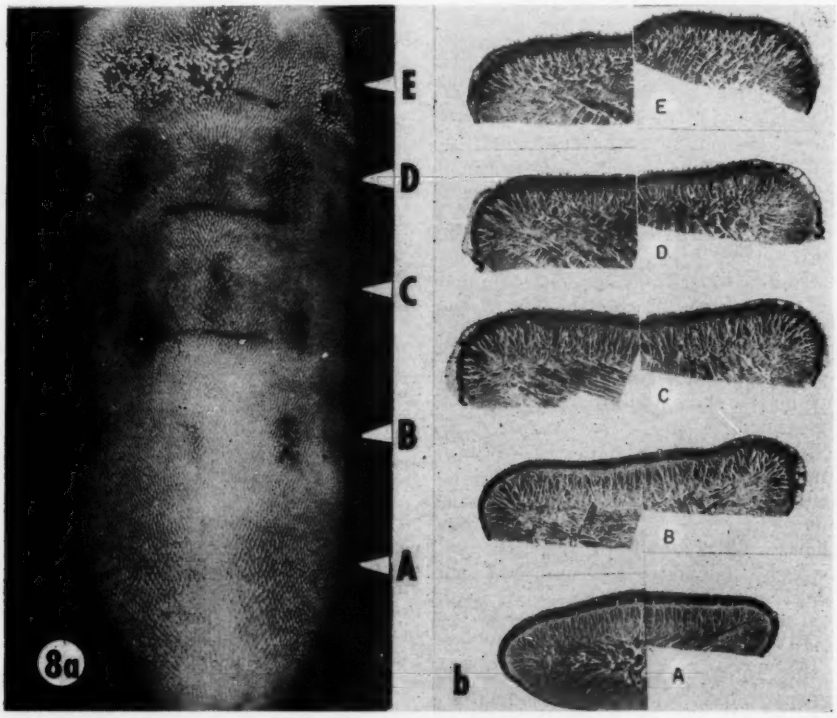

Fig. 8a—Photograph of bovine tongue two days postinoculation. Posible gross vesticulation is difficult to evaluate. The surface of nonvesiculated lesion areas is becoming dark and ragged from dehydration of the initial lesion and erosion (loss of some of the conical papillae).

Fig. 8b—Photomacrograph of sections from tongue shown in figure 8a. There is shallow gross vesiculation at C, left border, at D, left border, and at E, right border (collapsed vesicles). The nonvesiculated lesion is predominantly in a stage of dehydration and beginning erosion as indicated by the dense (although lighter than normal) appearance and ragged surface. Normal mucosa is shown at A and at B, left edge and middle dorsum.

method and the uncertainty in determining previously existing vesicles, it was evident that gross vesiculation had not developed to any extent beyond the second day postinoculation.

Nonvesiculated lesions seen in the photomacrographs and on low-power microscopic examination of the sections were generally more dehydrated than on the second day postinoculation (fig. 12b). Almost all lesions in tongues infected with New Jersey-type virus were quite dry (fig. 12b), undergoing maceration. Thus the subgross lingual pathologic changes at three days postinoculation were characterized by (1) erosions from previous vesiculation, but no additional vesiculation, and (2) dehydration with erosion or, in some instances, maceration of the mucosa in lesion areas where vesiculation had not occurred.

Four Days Postinoculation.—Gross vesicles were not in evidence, and no attempt was made to determine the percentage of (previous) gross vesiculation. All of the

Fig. 9—Photomicrograph of edge of typical gross vesicle. The necrotic-edematous mucosa has separated, almost in its entirety, from the underlying tissue. Portions of rete pegs and connective tissue papillae of the lamina propria remain attached to the floor of the vesicle. x 27.5.

and in some areas were beginning to erode by crumbling (fig. 13). Even more advanced erosion was observed in other tongues (not illustrated) infected with New Jersey-type virus. More than half of the nonvesiculated lesions in tongues infected with Indiana-type virus were dehydrated and showed beginning erosion by crumbling. The remaining lesions were still somewhat edematous and appeared to be original nonvesicular lesions showed partial or complete erosion. Portions of necrotic mucosa that remained attached to the tongue were dehydrated (fig. 14b). Many eroded areas showed a beginning of regeneration of the epithelium (fig. 15). There was less indication of epithelial regeneration where gross vesiculation presumably had taken place. Much destruction and loss of the basal epithelial cells was evident in

these areas. Several small, recent lesions were seen on the lateral borders of one tongue at A and on the dorsum at B. These lesions (not illustrated) were in the initial necrotic, edematous stage and had not undergone gross vesiculation. Presumably they resulted from delayed action of the highly diluted virus inoculated at B.

Gross Pathology.—The observations made on histopathologic and subgross examination give the pathologic-anatomical basis for the gross appearance of the lesions in the lingual mucosa. The initial (necrotic-edematous) lesions at two days postinoculation varied in gross appearance from barely perceptible to definite, slightly

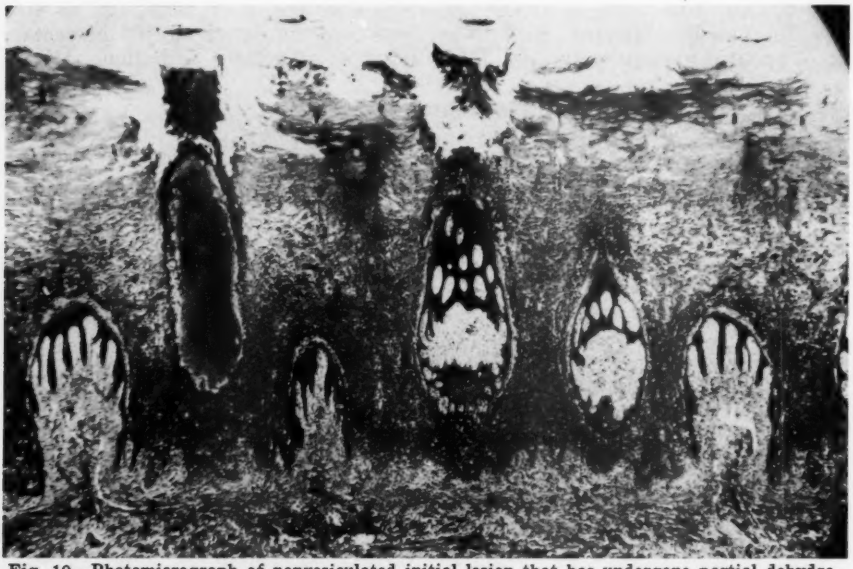

Fig. 10—Photomicrograph of nonvesiculated initial lesion that has undergone partial dehydration through microscopic breaks in the stratum corneum. x 27.5. Compare with figure 4.

Fig. 11—Photomicrograph of nonvesiculated initial lesion that has undergone complete dehydration through breaks in the surface, and is beginning to erode. x 27.5. Compare with figures 4 and 10.

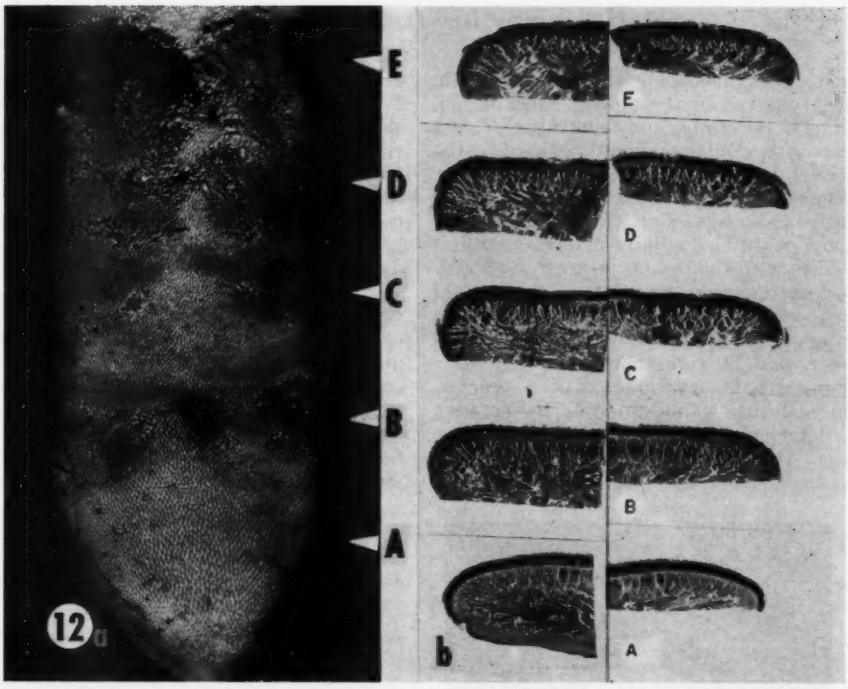

Fig. 12a—Photograph of bovine tongue three days postinoculation. Deep erosion indicative of previous vesiculation is seen at E, left and right borders. The large amount of nonvesiculated lesion appears darker than normal on account of dehydration of initial lesion. The ragged portions represent early erosion (loss of some conical papillae); the smooth areas represent more advanced erosion (loss of all the conical papillae).

Fig. 12b—Photomacrograph of sections of tongue shown in figure 12a. Deep erosion indicative of previous vesiculation is shown at D, left border and right border, and at E, left and right borders. All of the nonvesiculated lesion has undergone dehydration and in some areas is eroding by crumbling. Normal mucosa is shown only at A.

Fig. 13—Photomicrograph of a portion of a nonvesiculated lesion three days postinoculation. The lesion has progressed beyond the initial stage, has dehydrated completely and is eroding by crumbling. x 27.5.

raised and thickened, relatively smooth, blanched areas in the mucosa (fig. 5a, 6a, 7a). The swelling and blanching of the affected mucosa was evidently caused by the intercellular edema. Regardless of their perceptibility, the initial lesions gave a positive Nikolsky sign, *i.e.*, the diseased mucosa could be displaced laterally by firm pressure with the thumb. Unruptured vesicles had the gross appearance that would be expected of a vesicle (fig. 5a—B, left border; fig. 6a—B, right dorsum and border, and D, right border). They were circumscribed elevations of the mucosa, each enclosing a fluid-filled cavity between the detached mucosa and the underlying tissue. However, the smaller and characteristically more shallow vesicles [3, 9, 10] were difficult to distinguish grossly from well-developed initial lesions that were definitely elevated and blanched. A few vesicles had ruptured to produce deep, grayish red erosions with flaps of necrotic mucosa attached to the border (fig. 5a—E, left border)—the classical picture of a ruptured vesicle as illustrated in textbooks on animal diseases.

Areas of initial lesion that were dehydrating and beginning to erode, instead of undergoing vesiculation, were frayed and rough on the surface and darker in color than normal (fig. 7a—E, dorsum, and fig. 8a, C, D, E, dorsum). The frayed, rough appearance was caused by loss of the cornified tips of some of the conical papillae, along with erosion of the stratum corneum.

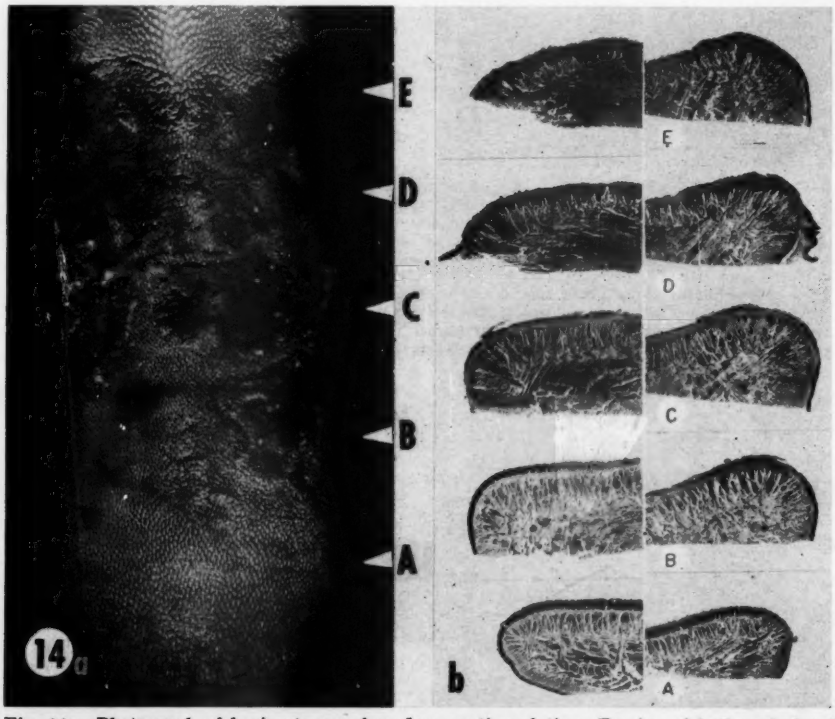

Fig. 14a—Photograph of bovine tongue four days postinoculation. Erosion of both vesiculated and nonvesiculated lesions has advanced to a point where estimation of previous vesiculation is no longer possible. The dark granular areas of D and E are areas where nonvesiculated lesions have eroded down to the bases of the conical papillae. Patches of necrotic mucosa are still adherent at B, right dorsum, and at C, left and right dorsum.

Fig. 14b—Photomacrograph of sections of tongue shown in figure 14a. Total erosion of the mucosa has occurred at D and E, almost complete erosion at C, and partial erosion at B, right side. Estimation of previous vesiculation as opposed to lesion that failed to undergo gross vesiculation is no longer possible. Normal mucosa is shown at A (except right border) and B, left side. Regeneration of epithelium on this tongue is shown in figure 15.

The darker coloration was an effect of necrosis and desiccation of the mucosal tissue.

Tongues observed on the third day postinoculation showed somewhat more advanced changes. Grossly evident vesicles were no longer apparent. Instead there were deeply eroded areas where vesicles presumably had developed and ruptured (fig. 12a—E, left and right borders). Initial lesions that had not undergone vesiculation were either frayed on the surface from partial erosion of conical papillae or smooth from complete loss of the conical papillae (fig. 12a—E, dorsum). The dark coloration was a result of necrosis and dehydration of the diseased mucosa.

On the fourth day postinoculation, fairly complete erosion of some of the nonvesiculated lesions had occurred, bringing into view the bases of the conical papillae (the connective tissue cores covered by a thin layer of regenerating epithelium) (fig. 14a—D, dorsum, and E, dorsum). These areas were granular in appearance because of the papillae and were darker than normal because of the thin, imperfect epithelial covering. Patches of necrotic mucosal debris remained attached, adding to the rough, irregular appearance of the lingual surface (fig. 14a—C, left, and B, right). Judging from the extent of the erosion and the progress being made in epithelial regeneration, it could be expected that healing would be rapid in areas not involved by secondary infection.

DISCUSSION

These observations on experimentally induced lesions of vesicular stomatitis in the bovine lingual mucosa are in practical agreement with clinical reports from the field. Even though gross vesiculation was evident in the experimental animals, the percentage of all initial lesions that became manifestly vesicular was low (less than 30%). Furthermore, gross vesicles that developed were evident only on the second day following inoculation. On the third and fourth days, the vesicles had terminated in deep erosions and the remaining large percentage of nonvesicular lesions were undergoing dehydration and erosion *in situ*.

In order to compare experimental and clinical observations, it must be assumed that lingual lesions from experimental infection with vesicular stomatitis virus and those from natural infection behave in the same way. Granting this assumption, an even lower percentage of observed lingual vesiculation would be expected in naturally infected cattle because, under field conditions, the examinations might be made at times when unruptured vesicles were not evident. Reports concerning failure or near failure to find vesicles in animals with vesicular stomatitis bear this out.[1, 11]

Observations on the nature and behavior of vesicular stomatitis lesions in the bovine lingual mucosa do not necessarily apply to lesions occurring in other areas such as the interdigital spaces or the teats. Nor do these observations necessarily apply to lingual lesions of vesicular stomatitis in other species. The bovine lingual mucosa is a unique anatomical structure. It is thick and firmly attached by the numerous, relatively stout, conical papillae. Consequently, intercellular edema of the bovine lingual mucosa is not enough to produce a lesion that is grossly vesicular in character. The

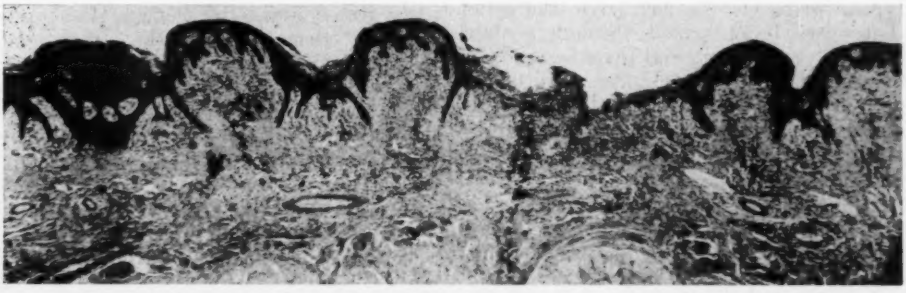

Fig. 15—Photomicrograph of a portion of a lingual lesion four days postinoculation showing complete erosion (loss) of the damaged epithelium and the beginning of epithelial regeneration. x 27.5.

anchoring effect of the conical papillae must be overcome by extensive epithelial necrosis and physical stress before the mucosa separates and a grossly evident blister develops. Thin-skinned areas devoid of such conical papillae would logically be more prone to gross vesiculation when involved by a pathologic process in which severe intercellular edema plays a part.

Certain of the histologic observations that were made suggest that another factor besides anatomical structure and physical stress may influence the development of gross vesicles. At the edge of some initial lesions severe intercellular edema was sometimes found without either epithelial necrosis or cellular infiltration. On the other hand, epithelial necrosis occurred in areas where edema was slight or absent. The apparent independence of intercellular edema and necrosis suggests that these pathological alterations represent two separate and distinct virus effects. Thus, they could be expected to occur in varying proportions depending on virus strain and host reaction. It follows, then, that strains of virus producing a combination of intercellular edema and necrosis most favorable to vesiculation would produce the most gross vesiculation, and *vice versa.*

It has been customary to refer to all epidermal and mucosal lesions of the vesicular diseases as vesicles. Lesions of the initial type that are elevated on gross examination and edematous on histologic examination have been classified as early, beginning, unripe, or microscopic vesicles (prevesicles). Initial lesions that have failed to vesiculate and have advanced to a dry, necrotic, and eroding stage have been regarded as ruptured or collapsed vesicles. The fact that some lesions develop into typical gross vesicles has led to the belief that every lesion passes through a stage of gross vesiculation, and that the inability to observe vesicles in clinical cases of the disease results entirely from failure to observe the case at the right time. The studies reported herein indicate that this belief is erroneous.

In an academic sense, the initial lesion that fails to undergo gross vesiculation could be classified as an abortive vesicle; namely, an edematous, necrotic lesion that failed to fulfill its destiny and develop to an actual vesicle. However, in a practical sense, the gross pathological characteristics of the vesicular disease are of the greatest importance because these diseases are diagnosed in the field by their gross appearance. It does not help the clinician's understanding of the disease, nor improve his diagnostic proficiency, when the histopathologist classifies as vesicular an initial lesion that does not appear to be vesicular on gross examination, or talks about microscopic vesicles and collapsed vesicles. A desirable approach would be to adhere to the principles of descriptive pathology. There is a need to work out gross, subgross, and microscopic correlation of lesions in different locations and in different species. The "vesicle" of the vesicular diseases promises to be a fruitful subject for investigation by both conventional and recently developed methods in pathology.

SUMMARY

A special technique was devised to study the morphogenesis of the lingual lesions of vesicular stomatitis in cattle.

The initial effect of vesicular stomatitis virus upon the bovine lingual mucosa was the development of blanched, slightly elevated lesions characterized microscopically by intercellular edema, necrosis of epithelial cells, and inflammatory cellular infiltration.

Less than 30 per cent of the total initial lesion on the tongues of 20 steers inoculated with two strains of the virus developed into grossly evident vesicles by separation of the diseased mucosa from the underlying tissues. All vesiculation occurred within 48 hours postinoculation. Vesicles were prone to occur at locations where there was friction between the tongue and the teeth or the dental pad.

The large percentage of initial lesions that failed to undergo gross vesiculation dehydrated *in situ,* by seepage of the intercellular edema through the stratum corneum, and eroded in the manner of a dry, necrotic, mucosal lesion. The total pathologic picture of the bovine tongues observed two, three, and four days postinoculation is described and illustrated.

These studies developed a revised concept of the pathologic changes of the tongue in cattle with vesicular stomatitis. The low percentage of vesiculation, as well as its transitory nature under controlled conditions of infection and observation, confirm clinical findings in recent field outbreaks of vesicular stomatitis in cattle.

XUM

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Quantities of 17-Hydroxycorticosteroids in the Plasma of Healthy Cattle During Various Physiologic States

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THE PRESENT emphasis on "stress" in relation to disease has resulted in a tendency to classify as "stress factors" many of the unfavorable environmental and management factors of farm animals without first obtaining evidence of measured changes of the function of the adrenal glands. Much has been learned by determination of the histopathologic alterations of adrenal tissue of healthy and diseased animals, but unfortunately this is not a method applicable to the measurement of the functions of the glands in the living animal.

For the recognition of a hypofunctional state of the adrenals the 17-hydroxycorticoids are the most satisfactory. However, there are serious limitations to the use of 17-hydroxycorticoids as relative indicators of the extent of overall adrenal steroidogenesis. The adrenal cortical steroid mixture is not entirely constant from day to day. In pathologic states other steroid fractions may increase markedly. Examples of this may be found in the elevated neutral 17-ketosteroids in the adrenogenital syndrome; in a rare form of Cushing's syndrome where there is a preponderance of corticosterone; and in the low potassium associated with sodium retention, in which aldosterone levels are found to be elevated. In spite of these deficiencies, 17-hydroxycorticoids and their hem-

TABLE 1—Quantities of 17-Hydroxycorticosteroids in the Plasma of Healthy Cattle

Physiologic status	No. of animals	No. of samples	17-OH-CS (μg./100 ml.) mean ± standard error
Calves (9 a.m.)	18	87	3.80 ± 0.21
Unbred heifers (9 a.m.)	6	83	4.39 ± 0.40
Unbred heifers (4:30 a.m.)	4	11	4.15 ± 0.65
Unbred heifers (3:30 p.m.)	4	11	3.09 ± 0.81
Unbred heifers in or near estrus (9 a.m.)	6	19	10.28 ± 1.24
Pregnant heifers (9 a.m.)	5	33	4.55 ± 0.76
Lactating nonpregnant cows (9 a.m.)	22	66	4.27 ± 0.46
Lactating cows in or near estrus (9 a.m.)	10	10	10.77 ± 1.64
Lactating pregnant cows (9 a.m.)	12	71	4.68 ± 0.37
Dry pregnant cows (9 a.m.)	13	22	4.28 ± 1.03
Labor (1–10 hours before)	12	12	4.01 ± 0.94
Immediately postpartum	14	14	3.04 ± 0.64
4–24 hours postpartum	12	12	4.55 ± 0.56

Forsham, in Williams' "Textbook of Endocrinology," [5] stated:

> The determination of the 17-hydroxycorticoids has the advantage of focusing on the anti-inflammatory and quantitatively largest fraction of the adrenal output. Furthermore, the methods available are the simplest of the steroid determinations. The 17-hydroxycorticoids are the best single metameter of overall adrenal cortical function. Since only about 10 per cent of the total, in the urine, is biologically active, most of the material consisting of the active di- and tetrahydro derivatives, there is little correlation between this chemical method (for urine) and bio-assays.

atologic metameter, the eosinophil count, are the most direct indicators of the overall state of adrenal cortical function. They must be supplemented by the measurement of other adrenal cortical derivatives in the presence of clinical symptoms suggestive of an imbalance in the usual mixture of corticoids.

Robertson and Mixner [4] suggested a method of measuring levels of 17-hydroxycorticosteroids (17-OH-CS) in the plasma of cattle, using a modification of the Porter-Silber reaction.[3] They reported that "blood samples were obtained from 23 dry, pregnant cows within the six-week period before parturition and from 20 milking, nonpregnant cows within the six-week period after parturition. The plasma 17-hydroxycorticosteroid values for the dry, pregnant group ranged from 6.90 to 17.62 with a mean

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value of 9.77 γ per cent, whereas the values for the milking, nonpregnant group ranged from 2.14 to 8.40 with a mean of 4.58 γ per cent. The increase in plasma 17-hydroxycorticosteroid levels accompanying pregnancy is in agreement with that in the human reported by Gemzell,[2] who found 100 per cent increase in the plasma steroid levels during pregnancy.'' Bailey[1] reported that certain diseases of dairy cattle were associated with evidence of increased levels of 17-OH-CS.

Obviously, additional information on healthy animals will be helpful.

TABLE 2—Relationship of Pregnancy and Plasma 17-Hydroxycorticosteroids in Cows

No. of months before calving	No. of animals	17-OH-CS (μg./100 ml.) mean ± standard error
9	16	3.92 ± 0.79
8	17	5.12 ± 0.92
7	16	5.02 ± 0.71
6	13	4.25 ± 0.76
5	10	4.12 ± 0.74
4	9	2.47 ± 0.81
3	9	5.35 ± 1.00
2	9	2.60 ± 0.82
1	9	3.10 ± 1.28

METHODS

With a slight modification of the Robertson and Mixner technique,[4] determinations of 17-OH-CS in the plasma of 46 healthy cattle in various physiologic states were made over a period of about 18 months. The modification consisted of using 1.0N sodium carbonate instead of 0.1N sodium hydroxide for alkaline washings. Reclamations of added 17-OH-CS with both methods are comparable (about 80%).

RESULTS AND DISCUSSION

Examination of the means and standard errors of the 451 measurements of 17-OH-CS of the plasma of the 46 cattle (table 1) indicates that with the exception of the values of the heifers and cows in or near estrus there were no significant differences in the values of the calves, unbred heifers, pregnant heifers, lactating nonpregnant cows, lactating pregnant cows, dry pregnant cows, cows in labor, and cows immediately and several hours postpartum.

Estrus or near-estrus of the heifers (10.28 μg./100 ml.) and of the lactating nonpregnant cows (10.77 μg./100 ml.) was associated with 17-OH-CS values of highly significant difference ($P = >0.01$) when compared to values of the heifers not in or near estrus (4.39 μg./100 ml.) and the lactating nonpregnant cows not in or near estrus (4.27 μg./100 ml.).

Values of the pregnant heifers (4.55 μg./100 ml.), pregnant lactating (4.68 μg./100 ml.) and pregnant dry (4.28 μg./100 ml.) cows were not significantly different from the nonpregnant heifers (4.39 μg./100 ml.) and nonpregnant lactating cows (4.27 μg./100 ml.).

Values of lactating pregnant cows (4.68 μg./100 ml.) were not significantly different from dry pregnant cows (4.28 μg./100 ml.).

No significant diurnal variations were ascertained in the unbred non-estrual heifers at the three different times of sampling.

No particular pattern was apparent among monthly values of pregnant cows of this group (table 2).

SUMMARY

A total of 451 determinations of 17-hydroxycorticosteroids were made on the plasma of 46 cattle in various physiologic states. With the exception of estrual values of heifers and cows, no significant differences in plasma 17-OH-CS levels were observed.

No significant diurnal variations were ascertained in unbred non-estrual heifers.

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A Killed Vaccine Against Paratuberculosis (Johne's Disease) in Sheep

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A FIELD TRIAL of paratuberculosis (Johne's disease) vaccine in sheep has been in progress in Iceland for about eight years. The vaccine consists of killed *Mycobacterium paratuberculosis* suspended in mineral oil; it is injected subcutaneously.

Conditions for carrying out a field trial of paratuberculosis vaccine in Iceland were favorable during this period because, when the experiment was started, the incidence of paratuberculosis in certain areas was heavy. The difference in mortality between effectively vaccinated sheep and corresponding control groups could, therefore, be expected to be pronounced.

The reason for the heavy incidence of paratuberculosis in Iceland appears to be that this infection was not present in the sheep population there until recently. Once introduced, it encountered an immunologically virgin sheep population, resulting in a severe epizootic.

Paratuberculosis was brought to Iceland in 1933 when 20 sheep were imported from Germany for breeding purposes. These 20 sheep were distributed to 16 farms scattered over the country. Five years later, paratuberculosis was discovered for the first time in Iceland on one of these farms and at about the same time infection was found also on four more of the 16 farms where the rams had been introduced. It may thus be stated that at least 5 of the 20 sheep imported from Germany were carriers of paratuberculosis, although the disease had not been considered to be prevalent in sheep in that country.

On arrival, the imported sheep appeared to be in perfect health; it seems that the infection in all these sheep was, and probably always had been, completely "silent."

Paratuberculosis was first discovered in Iceland in 1938, and by 1939 it was clear that five separate foci of infection had been established. The disease spread slowly but continuously from the original foci and, when this experiment was started in 1950 and 1951, areas with heavy incidence could be selected in eastern Iceland.

Experimental work to develop a noninfective (killed) paratuberculosis vaccine had been carried out for a period of years before the present experiment was begun. Heat-killed and vacuum-dried *Myco. paratuberculosis* suspended in sterile mineral oil had been injected subcutaneously in quantities varying from 0.37 to 300.0 mg. per dose. The titer of complement-fixing antibody, the size of the local lesions at the site of the injection, and skin allergy to avian tuberculin had been followed monthly for 12 to 20 months.[1,2] On the basis of these findings, it was decided to use 5.0 mg. of dried bacteria per dose.

This type of vaccine was tested in a small field experiment with sheep to see whether it would protect lambs against infection in heavily infected flocks. In the fall of 1947, 1948, and 1949, approximately half of the lambs in each of six infected flocks, a total of 289 animals, were vaccinated.[3] The remaining 266 lambs were left as unvaccinated controls. These lambs were observed until they were killed in the fall of 1951. The intestines of sheep dying during this period were examined for the presence of *Myco. paratuberculosis*. Sixteen unvaccinated animals died from paratuberculosis during the period, but none from the vaccinated group.

At the end of the experimental period, the surviving sheep (225 vaccinated and 194 unvaccinated control animals) were necropsied and the intestines and mesenteric glands were carefully examined macro- and microscopically. Lesions of paratuberculosis were found in 21 sheep of the unvaccinated group and in 4 of the vaccinated group.

From the Institute for Experimental Pathology, University of Iceland, Keldur, Reykjavík, Iceland. Dr. Sigurdsson died during the summer of 1959.

The author thanks Mr. Glúmur Björnsson and Mr. Halldór Grímsson, fil. mag., for their help with the statistical evaluation of the material.

The author also acknowledges the tireless work of many people who have worked on this project: Mr. Gudmundur Egilsson who traveled for several winters through the experimental districts, often under adverse conditions; field inspectors Hermann Jónsson, Jón Gudmundsson, Sigurdur Pálsson, Sigurjón Sigurdsson, Alfred Pétursson, and Páll Sigurdsson; and laboratory assistants Miss Anna Tryggvadóttir and Miss Helga Helgadóttir, as well as several others.

It could be concluded that vaccination had not only prevented deaths from paratuberculosis but had also reduced the incidence of clinically unsuspected anatomic lesions.

When this experiment had been in progress for three years and it had become evident that the vaccine had provoked resistance to infection, a second field experiment on a much larger scale was initiated.

This experiment and early results obtained in it have already been briefly described.[4] The present report describes the plan of the experiment in more detail and gives the results obtained during the first six years of the experiment. It is planned to continue this study as long as an appreciable number of the experimental sheep survive.

PREPARATION OF VACCINE

A mixture of two old bovine strains of *Myco. paratuberculosis* was employed for the preparation of the vaccine. One of these was the so-called "Teps" strain which was obtained many years ago from Dr. R. E. Glover, then of the National Institute for Medical Research Farm Laboratories, Mill Hill, London. The other strain was called "strain 18" and was obtained from Dr. B. T. Simms, then of the Animal Disease Research Laboratory of the Bureau of Animal Industry, Auburn, Ala. The bacteria were cultivated on the surface of a liquid medium of the following composition: potassium phosphate, secondary 1.0 Gm.; feric citrate, 0.06 Gm.; magnesium sulfate, 2.0 Gm.; sodium citrate, 0.5 Gm.; glycerol, 70.0 ml.; asparagine, 5.0 Gm.; dried *Mycobacterium phlei*, 0.3 Gm.; distilled water, 1,000 ml.

The medium was distributed in 500-ml. Erlenmeyer flasks, 200 ml. in each, and autoclaved at 15 lb. for 20 minutes.

When the cultures were fully grown, usually after about eight weeks, they were harvested, filtered off, and put into test tubes 20 mm. in diameter and 170 mm. long. The tubes were about half-filled and immersed about four fifths of their length in a water bath of 70 C. for one hour. After this, the bacteria were placed in Petri dishes and dried in a vacuum oven at 40 to 45 C. Then the bacteria were weighed, ground in a mortar, and suspended in sterile mineral oil containing 0.3 per cent phenol. The concentration was so adjusted that each milliliter of vaccine contained 5.0 mg. of dried bacteria—2.5 mg. of Teps strain, and 2.5 mg. of strain 18.

The mineral oil was sterilized in a hot air oven at 150 C. for two hours. The phenol was added after the oil had been sterilized.

The sterility of the bacterial mass was carefully tested both before it was heated and after it was dried.

This procedure is still used for large-scale production of vaccine, with two changes: A light mineral oil (Bayol F, Esso Standard Oil Co., N.J.) is now used as a suspending medium and a colloid mill of our own construction with continuous feeding is now used to suspend the bacterial mass in the mineral oil. This gives a more homogeneous preparation.

PLAN OF PRESENT EXPERIMENT*

When the experiment was planned in 1950, paratuberculosis of sheep was causing extremely heavy losses in certain areas in eastern Iceland. It had been spreading for the past 15 years, slowly at first but more rapidly in later years and had become thoroughly seeded on most farms in certain areas; losses were, in fact, so heavy that sheep farming was seriously threatened in some districts.

Four districts in this area were selected for testing the vaccine because paratuberculosis was present on most of the farms in all the areas. It had been prevalent on some farms for several years but was recently introduced on others. Losses were heavy, so a vaccination procedure under such circumstances would be put to a severe and critical test.

Half of the lambs on 141 farms were vaccinated in the autumns of 1950 and 1951. The other half of the lambs on these same farms were kept as unvaccinated controls. All were marked with numbers and carefully recorded aluminum ear tags. In this way, direct comparison of the fate of vaccinated and unvaccinated lambs of the same age was made possible. This was considered important,

* *1950 group.*—Experimental lambs born in May and June, 1950; half of them were vaccinated in November and December of that year.

1951 group.—Experimental lambs born in May and June, 1951; half of them were vaccinated in November and December of that year.

Specimen group.—Group from which specimens were received—vaccinated or control animals dead before Dec. 31, 1956, and from which specimens of intestine were available for microscopic study.

No-specimen group.—Group from which specimens were not received—vaccinated or control animals dead before Dec. 31, 1956, from which no specimens were available for microscopic study.

Specific deaths.—Deaths from paratuberculosis, confirmed by microscopic examination of intestine.

Nonspecific deaths.—Deaths from causes other than paratuberculosis; in these cases, specimens of intestine were received but no microscopic evidence of paratuberculosis was found.

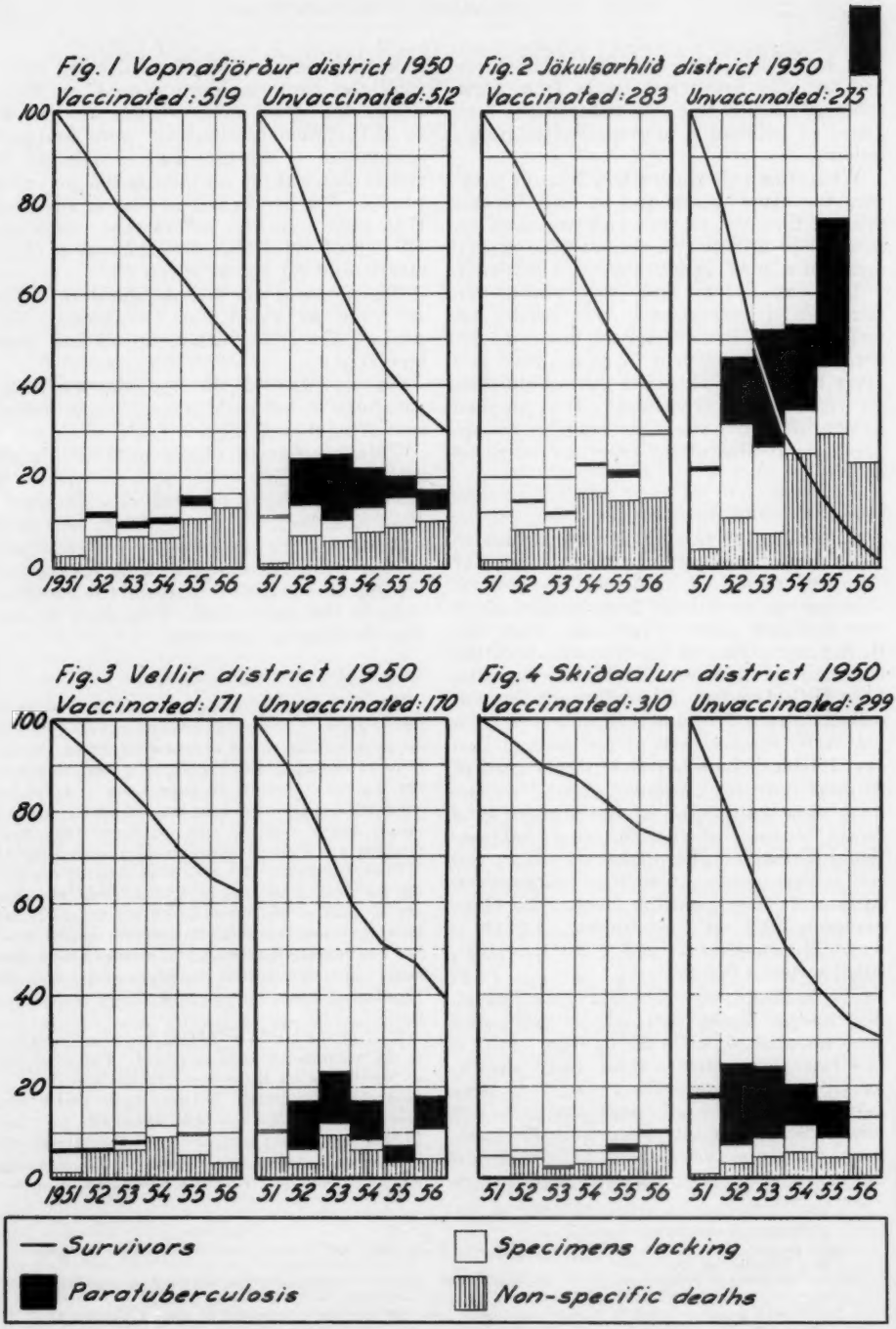
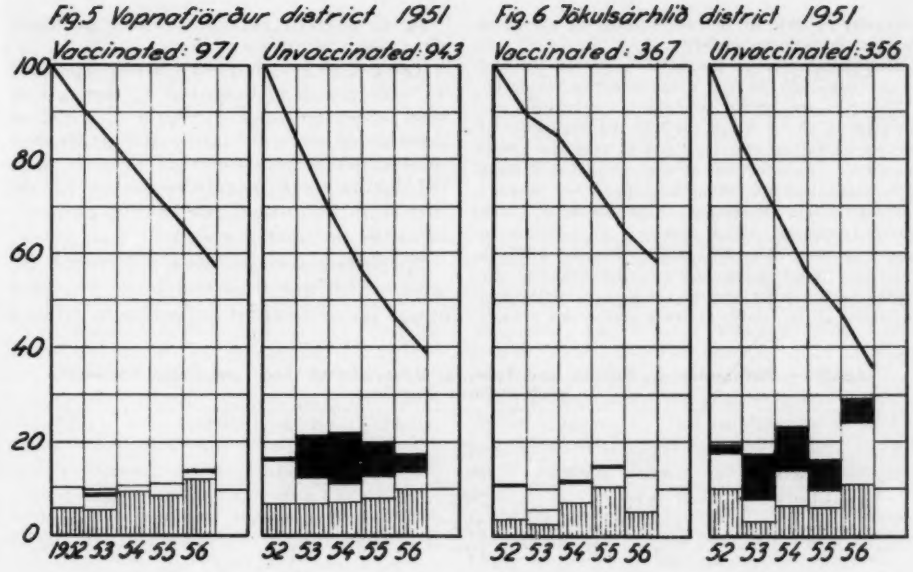
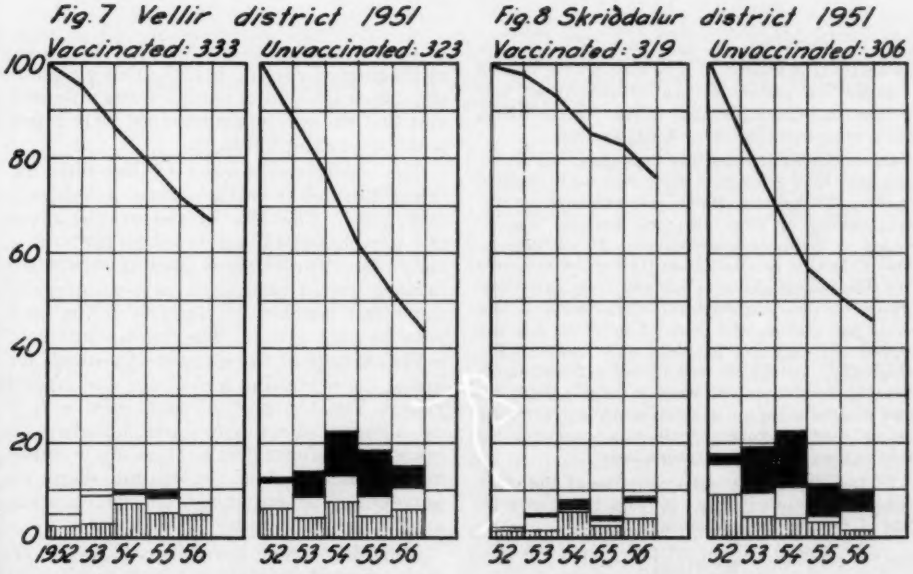


Fig. 1 Vopnafjörður district 1950
Vaccinated: 519
Unvaccinated: 512
Fig. 2 Jökulsarhlið district 1950
Vaccinated: 283
Unvaccinated: 275
Fig. 3 Vellir district 1950
Vaccinated: 171
Unvaccinated: 170
Fig. 4 Skíðdalur district 1950
Vaccinated: 310
Unvaccinated: 299
100
80
60
40
20
0
1951 52 53 54 55 56
51 52 53 54 55 56
— Survivors
Paratuberculosis
Specimens lacking
Non-specific deaths





Figures 1 to 8 show the results of the vaccination experiment by district and year-group. The columns show annual mortality from paratuberculosis and from other causes. The line shows the number of sheep surviving at the end of each year, calculated as a percentage of the number of sheep in each group at the beginning of the experiment. In one instance (fig. 2) the mortality exceeds 100 per cent, due to the method of calculation (see text).

primarily because of the well-known age variation in paratuberculosis mortality.

The distribution of the animals, vaccinated and unvaccinated, in the four areas is shown (table 1).

The farmers agreed to collect information regarding all sheep which died in the experimental groups, as far as practical, and to take two specimens from the intestines of each animal, put them in formalin, and keep them until they were collected and sent to our laboratory. Also, one of our men traveled through these areas from autumn to spring in 1951–1952, 1952–1953, and 1953–1954, in order to collect specimens and information. He visited each farmer several times each winter and examined all the sheep at least once every year.

TABLE 1—Distribution, by Districts and Years, of Unvaccinated Sheep and Sheep Vaccinated Against Paratuberculosis

Districts	1950		1951	
	Vaccinated	Controls	Vaccinated	Controls
Skriddalur	310	299	319	306
Jökulsárhlíd	283	275	367	356
Vellir	171	170	333	323
Vopnafjördur	519	512	971	943
Totals	1,283	1,256	1,990	1,928

During 1954–1955 and 1955–1956, the control in the field was entrusted to four local people trained to follow the pattern of control already set, one of them in each of the four areas. On the whole, this arrangement has proved satisfactory.

All specimens of intestines were examined microscopically soon after they were received in the laboratory. If typical *Myco. paratuberculosis* was seen, usually in large numbers, the case was recorded as being paratuberculosis. If the description of the case received from the farmer indicated that the sheep might have suffered from paratuberculosis but no mycobacteria were found in the smear preparation, the piece of intestine was cut, stained for acid-fast bacteria, and examined histologically. If typical lesions were found under these circumstances, which was only rarely, the case was recorded as paratuberculosis. If typical lesions were not found, death was considered not to have been due to paratuberculosis.

To facilitate statistical evaluation of the material, a punch-card system was used to register the fate of the experimental sheep.

RESULTS

Although this field experiment will be continued for several more years, the results obtained through 1956 will be reported here. The sheep in the experimental groups had then reached an age when paratuberculosis mortality had declined greatly, as will be shown. It seems unlikely, therefore, that the present results will change much after final returns are in.

Table 2 shows that of 6,457 sheep 3,493 were recorded as dead before the end of 1956. Specimens of intestine of 2,302 of these sheep were examined and paratuberculosis lesions were found in 781. Of these 781 deaths due to paratuberculosis, 722 occurred in the unvaccinated group and only 59 in the vaccinated group.

Specimens were not received from 1,191 sheep which were reported dead. The ratio of specimens received to specimens missing in the vaccinated group was 1:1.90; in the unvaccinated group, 1:1.95. This indicates that the collection of samples was unbiased, and that the specimens received were representative of the whole.

The mortality in each of the four districts through 1956 is shown graphically (fig. 1–8). The line in the graphs shows the percentage of survivors at the end of each year. The columns show the mortality in each group calculated as percentage of the mean number of animals living each year in each group. The "mean number" is the average of the number of animals living at the beginning and at the end of each year.* The top part of each column indicates known paratuberculosis mortality, the lowest part shows deaths from other causes, and the mid-part of the column shows the percentage of animals from which specimens were not received.

Mortality from paratuberculosis in the unvaccinated animals is heavy in all areas but is much lower, almost negligible, in the

* Mortality in this report is calculated from the mean number of the population in a given period rather than from the initial number. This method is used in order to avoid distortion in the mortality figures due to reduction in population number from causes other than the one under consideration each time. This would have happened, for example, in table 5.

vaccinated groups (fig. 1–8). This is equally true of all the areas.

Another point worth stressing is that total mortality, as reflected in the line in the graphs, is also much heavier in the unvaccinated groups. This is, of course, partially explained by the difference in recognized paratuberculosis mortality in the two groups. It seems, for reasons to be explained later, that paratuberculosis is probably responsible for most of the remaining excess mortality in the nonvaccinated group.

The mortality observed in the various districts during the whole period up to Dec. 31, 1956, is calculated as the mean number of deaths in percentage of the mean population each year (fig. 9). Clearly, specific mortality and total mortality are much heavier in unvaccinated than in vaccinated sheep, but *nonspecific mortality in animals from which specimens were received* (bottom part of each column) is closely similar in vaccinated and nonvaccinated sheep. On the other hand, the groups from which specimens were not received (mid-part of column) are much larger on the unvaccinated side.

TABLE 2—Deaths from All Causes in Vaccinated and Unvaccinated Sheep from Beginning of Experiment through 1956

Item	No. of sheep at beginning of experiment	No. of deaths	Specimens of intestine		Deaths from paratuberculosis
			Received	Missing	
Vaccinated	3,273	1,393	913	480	59
Unvaccinated	3,184	2,100	1,389	711	722
Total	6,457	3,493	2,302	1,191	781

Tables 3 and 4 show, in columns 3 and 4, the total number of deaths and the total mortality each year up to the end of 1956 in both group 1950 and group 1951. Column 5 shows from what percentage of dead animals intestinal specimens were received for microscopic study; columns 6, 7, 8, and 9, the results of the microscopic examination of specimens received; columns 10 and 11, the number and percentage of dead animals from which specimens were not received.

It was mentioned earlier in this report that the ratio of number of sheep from which specimens were received to number of sheep from which specimens were not received is similar in the vaccinated (1:1.90) and in the unvaccinated (1:1.95) groups. The animals from which specimens were received thus appear to be representative of the whole. An estimate of the total mortality due to paratuberculosis in both groups from 1950 and 1951 can, therefore, be calculated.

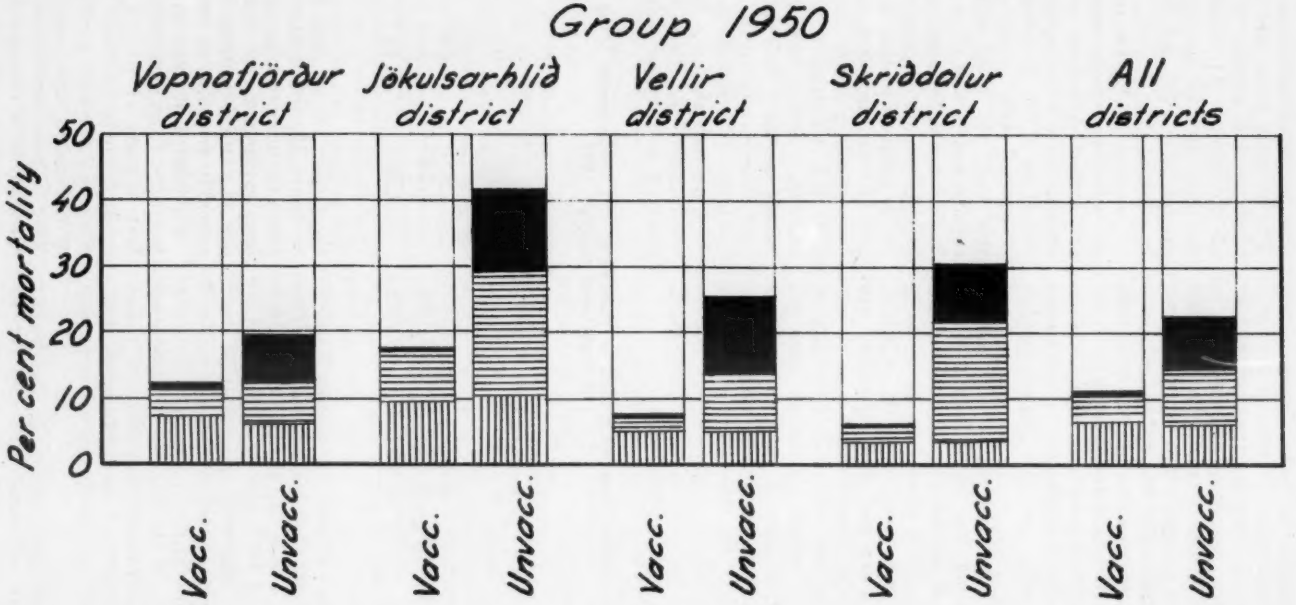
Columns 12, 13, 14, and 15 (tables 3, 4) are calculated on the assumption that paratuberculosis killed the same proportion of animals from which specimens were not received as it did in the group from which specimens were received. Calculated in this way, the total mortality from nonspecific causes was of course again similar in the vaccinated and control groups. The ratio of calculated paratuberculosis mortality in controls to that in vaccinated sheep is 1:14.9 for the 1950 groups (table 3). The corresponding figure for the 1951 groups is 1:16.4. Calculated in this way the vaccination is seen to have reduced paratuberculosis mortality 93.3 per cent and 93.9 per cent, respectively, in the two groups.

Figure 10 presents in a more synoptic form the data shown in figure 9. As before, the animals from which specimens were not received are represented in the graph by the midsection of each column. However, in figure 10, this midsection has been divided to indicate what proportion of it has been calculated (*see* tables 3 and 4) to be due to paratuberculosis and what part has probably been due to other causes.

Calculated in this way, the mean annual mortality from paratuberculosis during the first 6 years of life in the unvaccinated group born in 1950 was 11.9 per cent, but it was 8.2 per cent during the first 5 years of life in the unvaccinated sheep born in 1951. These figures are an indication of the severity of the epizootic with which the vaccine had to contend.

Information was collected from the farmers about the cause of death of each

Fig. 9—Results are shown from individual districts. The mean annual mortality is calculated as a percentage of the mean number of living sheep during the whole experimental period.



Group 1951
Per cent mortality
50
40
30
20
10
0
Vopnafjörður district
Jökulsárhlíð district
Vellir district
Skriðdalur district
All districts
Vacc.
Unvacc.
Paratuberculosis deaths
Specimens lacking
Nonspecific deaths

TABLE 3—Fate of Experimental Sheep, Born in 1950, in the Period from 1950 to Dec. 31, 1956

Year	Mean population	Dead from all causes		Specimens received as percentage of dead sheep	Specimens received				Specimens not received		Calculated nonspecific deaths		Calculated paratuberculosis deaths	
					Not paratuberculosis		Paratuberculosis							
		No.	%		No.	%	No.	%	No.	%	No.	%	No.	%
VACCINATED														
1951	1,227.5	111	9.0	19.8	21	1.7	1	0.1	89	7.2	106	8.6	5	0.4
1952	1,113.0	118	10.6	67.8	73	6.6	7	0.6	38	3.4	108	9.7	10	0.9
1953	1,011.0	86	8.5	77.9	60	6.0	7	0.7	19	1.9	77	7.6	9	0.9
1954	913.5	109	11.9	70.6	72	7.9	5	0.5	32	3.5	102	11.2	7	0.8
1955	805.5	107	13.3	75.7	70	8.7	11	1.4	26	3.2	92	11.4	15	1.9
1956	701.5	101	14.4	71.3	71	10.1	1	0.1	29	4.1	100	14.3	1	0.1
Total	5,772.0	632	10.9	63.1	367	6.3	32	0.6	233	4.0	585	10.1	47	0.8
UNVACCINATED														
1951	1,168.5	175	15.0	16.0	22	1.9	6	0.5	147	12.6	137	11.7	38	3.3
1952	950.0	262	27.6	70.6	62	6.5	123	12.9	77	8.1	88	9.3	174	18.3
1953	716.0	206	28.8	78.2	46	6.4	115	16.1	45	6.3	59	8.2	147	20.6
1954	546.5	133	24.4	77.4	50	9.2	53	9.7	30	5.5	65	11.9	68	12.5
1955	434.0	92	21.2	72.8	35	8.1	32	7.4	25	5.8	48	11.1	44	10.1
1956	352.5	71	20.2	60.6	29	8.2	14	4.0	28	8.0	48	13.6	23	6.5
Total	4,167.5	939	22.5	62.5	244	5.9	343	8.2	352	8.4	445	10.7	494	11.9

The figures in column 12 are the sum of the numbers in column 6 and a proportional fraction of the numbers in column 10. Column 14 is obtained similarly by adding the numbers in column 8 and the rest of the numbers in column 10 (see text).

TABLE 4—Fate of Experimental Sheep Born in 1951 in the Period from 1951 to Dec. 31, 1956

Year	Mean population	Dead from all causes		Specimens received as percentage of dead sheep	Specimens received				Specimens not received		Calculated nonspecific deaths		Calculated paratuberculosis deaths	
					Not paratuberculosis		Paratuberculosis							
		No.	%		No.	%	No.	%	No.	%	No.	%	No.	%
VACCINATED														
1952	1,910.0	160	8.4	50.0	79	4.1	1	0.1	80	4.2	158	8.3	2	0.1
1953	1,762.5	135	7.7	55.6	69	3.9	6	0.3	60	3.4	124	7.1	11	0.6
1954	1,612.0	166	10.3	82.5	128	8.0	9	0.6	29	1.8	155	9.6	11	0.7
1955	1,454.0	150	10.3	75.3	106	7.3	7	0.5	37	2.5	141	9.7	9	0.6
1956	1,304.0	150	11.5	72.7	105	8.1	4	0.3	41	3.1	144	11.1	6	0.5
Total	8,042.5	761	9.5	67.5	487	6.1	27	0.3	247	3.1	722	9.0	39	0.5
UNVACCINATED														
1952	1,784.0	288	16.1	54.5	136	7.6	21	1.2	131	7.3	249	14.0	39	2.2
1953	1,499.5	281	18.7	72.2	79	5.3	124	8.3	78	5.2	109	7.3	172	11.5
1954	1,223.0	272	22.2	76.5	81	6.6	127	10.4	64	5.2	106	8.7	166	13.6
1955	995.5	173	17.4	77.5	61	6.1	73	7.3	39	3.9	79	7.9	94	9.5
1956	840.5	147	17.5	68.0	66	7.9	34	4.0	47	5.6	97	11.6	50	5.9
Total	6,342.5	1,161	18.3	69.1	423	6.7	379	6.0	359	5.7	640	10.1	521	8.2

The figures in column 12 are the sum of the numbers in column 6 and a proportional fraction of the numbers in column 10. Column 14 is obtained similarly by adding the numbers in column 8 and the rest of the numbers in column 10 (see text).

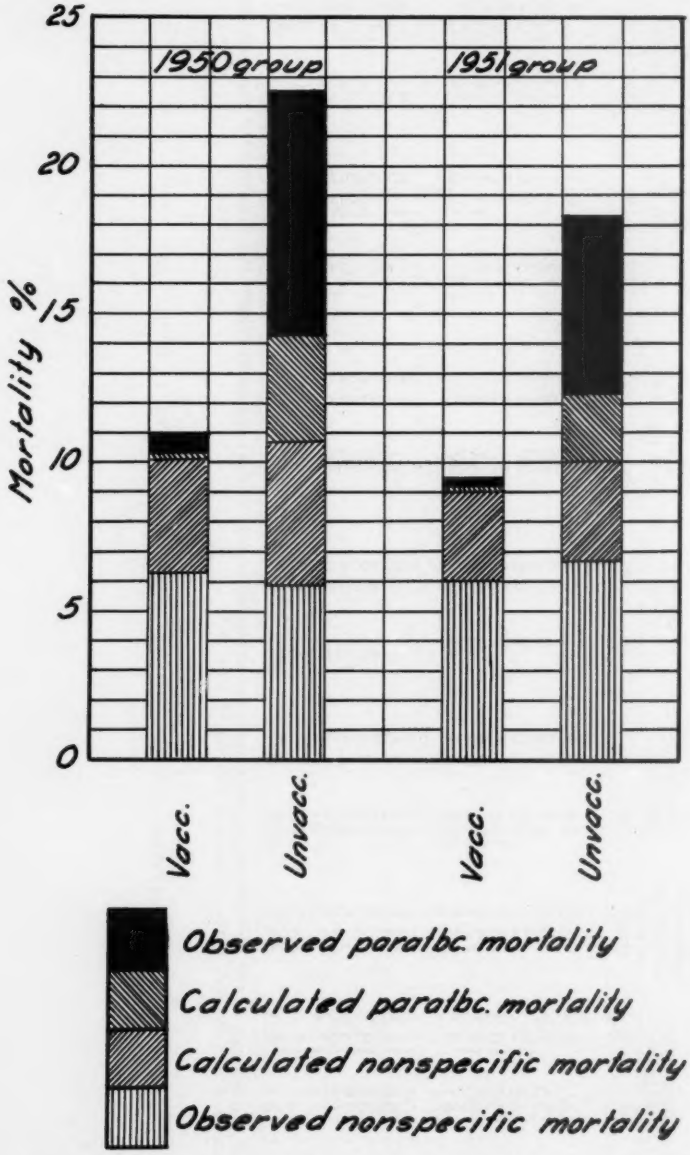


Fig. 10—Results are shown for all experimental districts taken together. The mean mortality per year is calculated as the mean number of deaths in percentage of the mean number living.

animal which died, irrespective of whether a specimen was received from it. Such information is admittedly incomplete but nevertheless it has considerable value. Deaths were classified as follows: (1) known infections other than paratuberculosis; (2) accidents; (3) slaughtered healthy; (4) missing; (5) cause uncertain, (6) slaughtered in poor condition. When mortality from these various causes (in the vaccinated and in the unvaccinated groups on the one hand and in the groups from which specimens were received on the other hand) was compared, certain interesting facts emerged.

If one considers the reported causes of death in the no-specimen groups, for example, it is seen that mortality due to

causes 1, 2, and 3 is similar in the vaccinated and control groups but that mortality due to causes 5 and 6 is much higher in the unvaccinated groups.

Table 5 shows this difference after the causes of death have been grouped together as was suggested in the preceding sentence. If paratuberculosis caused an appreciable mortality in the no-specimen groups, the two last causes listed in the table would be the ones most likely to mask such unrecognized cases. The evidence indicates that such is the case.

The number of deaths due to paratuberculosis and the mortality (in %) in each group varies with the age of the animals (columns 14 and 15, tables 3 and 4). It will be noticed that paratuberculosis mortality is highest during the third year of life except in one vaccinated group where the number is quite low and the highest mortality occurs during the fifth year of life.

This paratuberculosis mortality is plotted logarithmically (fig. 11), the 1950 and the 1951 groups being given separately. It is interesting to observe that the paratuberculosis mortality in the 1950 group is about 50 per cent higher than the paratuberculosis mortality in the 1951 group for five consecutive years. It should be remembered that both groups are on the same farms, living under the same conditions.

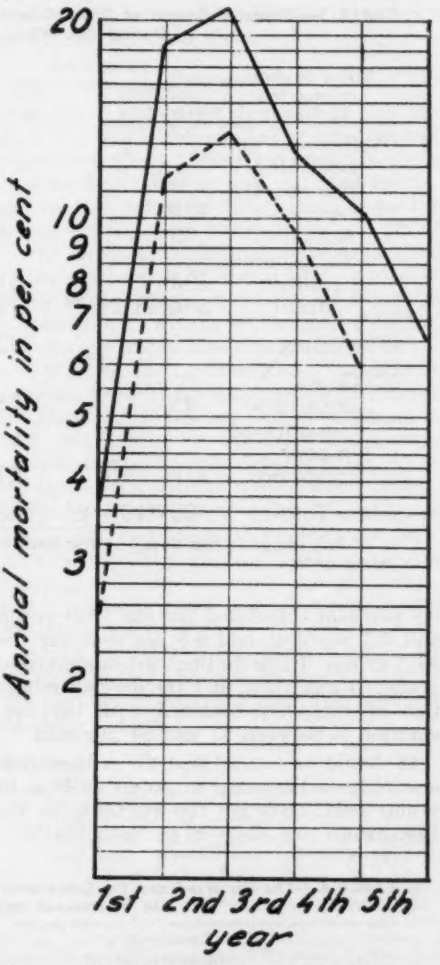


Fig. 11—The total calculated paratuberculosis mortality in unvaccinated sheep by years of age is shown. The solid line represents the 1950 group and the broken line represents the 1951 group. The scale is logarithmic.

DISCUSSION

The field trial of paratuberculosis vaccine here described has now lasted for about eight years. Only the results obtained up to the end of 1956 are considered in this report as it takes considerable time to obtain and analyze them. However, it seems clear that the results in 1957 and 1958 continue along the same line.

The present paper should be considered a preliminary report covering the first six years of the experiment but it seems unlikely that later experience with these flocks will do much to change the findings in principle as the peak mortality was passed in 1953 and in 1954, in the 1950 group and the 1951 group, respectively.

The data shown (tables 3, 4) and summarized (table 6) show that specific mortality in the uninoculated control groups was heavy. The observed average annual mortality from paratuberculosis was 8.2 per cent in the unvaccinated group from 1950 and 6.0 per cent in the unvaccinated group from 1951. Evidence is presented to indicate that the animals from which intestinal samples were received are a representative sample of all the animals which died during the experimental period. Assuming this to be so, the annual paratuberculosis mortality was 11.9 per cent in the unvaccinated group from 1950 and 8.2 per cent in the unvaccinated group from 1951. The corresponding figures for the vaccinated sheep are 0.6 per cent observed and

TABLE 5—Reported Causes of Death Other than Paratuberculosis in All Experimental Sheep During the Whole Period of Observation

Cause of death as reported by farmer	Specimens (vaccinated sheep)		Specimens (unvaccinated sheep)	
	Received	Lacking	Received	Lacking
(A) Known infections other than paratuberculosis.	222	56	118	67
Accidents.	90	105	71	106
Slaughtered healthy.	284	51	212	78
Total	596(23.2%)*	212(8.2%)	401(20.4%)	251(12.8%)
(B) Missing	.....	222(8.6%)	.....	291(14.8%)
(C) Cause uncertain.	45	25	44	95
Slaughtered in poor condition.	214	21	222	74
Total	259(10.1%)	46(1.8%)	266(13.5%)	169 (8.6%)

* Percentage is calculated on the basis of the average number of sheep during the whole period of observation.

0.8 per cent calculated for the 1950 group and 0.3 per cent and 0.5 per cent for the 1951 group. These findings are summarized (table 6) and show that the over-all reduction of mortality obtained with the vaccination is between 93 and 94 per cent.

It should be noticed that the reduction in mortality is the same in group 1950 as in group 1951, although the mortality in the 1950 group was about 50 per cent heavier.

However, the following possibility might perhaps also be considered: If we assume, as seems likely, that animals are usually infected with paratuberculosis during the first year of life, even in cases when the clinical disease appears much later, the risk

TABLE 6—The Observed and the Calculated Average Annual Mortality from Paratuberculosis in Vaccinated and Unvaccinated Sheep

Group	Observed mortality(%)		Calculated mortality(%)		Reduction in mortality(%)
	Vaccinated	Controls	Vaccinated	Controls	
1950	0.6	8.2	0.8	11.9	93.3
1951	0.3	6.0	0.5	8.2	93.9

The protection afforded by the vaccination is shown as the percentage of reduction in mortality observed in the vaccinated animals compared with the controls.

The remarkably regular difference from year to year in paratuberculosis mortality between the 1950 and 1951 groups is interesting when plotted (fig. 11).

No definite explanation for this difference can be offered, particularly as the "normal" annual variation in paratuberculosis mortality over a longer period is not known. The observed difference might be due to an annual environmental variation of unrecognized nature, such as a difference in the quality of feeds or varying weather conditions.

to which the animal is exposed would be in direct proportion to the amount of infective material in the environment during the first year. If we further assume that the greater part of infective *Myco. paratuberculosis* in the environment at any given time is contributed by animals of 1 to 2 years of age, *i.e.*, sheep with a recent, flowering infection, then the difference in

mortality between the 1950 and the 1951 groups might perhaps be explained as follows:

The 1950 group acquired its infection mainly from sheep born in 1948 and 1949, none of which had been protected by vaccination. The 1951 group acquired its infection mainly from sheep born in 1949 and 1950. Half of the population born in 1950 was, however, protected by vaccination and thus did not excrete *Myco. paratuberculosis* bacteria in appreciable quantity. According to this reasoning, the amount of infective material in the environment would, therefore, have been lower in 1951 (perhaps only ¾ of what it was in 1950) and this might explain the difference in the risk to which the two age groups were exposed.

At any rate, it is suggested (fig. 11) that the 1951 group was exposed to a risk so much lower than the 1950 group that its mortality from paratuberculosis remained constantly about one third lower than that of the 1950 group for at least five years.

However, it seems just as likely that the observed difference between age groups is caused by unrecognized environmental factors as has already been mentioned.

The vaccination procedure described in this paper produces local lesions at the site of injection which last for a long time. This is a nuisance and reduces the market value of the carcass. Although mutton from adult sheep is not an important market product, and although the real value of the carcass is not seriously lowered by these lesions, the damage to the appearance of the carcass is a drawback.

About 450,000 sheep have been vaccinated with this new type of vaccine during the past seven years and the protection given has been excellent.

SUMMARY

A field experiment designed to test the protective value of a killed vaccine against paratuberculosis (Johne's disease) in sheep is described. The experiment was carried out on 141 farms in four districts having heavy incidence of paratuberculosis. Half of the lambs on each farm were vaccinated in the autumns of 1950 and 1951 (a total of 3,273 lambs) and the other half were left unvaccinated as controls (a total of 3,184). All groups were closely followed up to the end of 1956. In the 1950 group, vaccination reduced the average annual specific mortality from 11.9 to 0.8 per cent, a reduction of 93.3 per cent. In the 1951 group, the specific mortality was reduced from 8.5 to 0.5 per cent, a reduction of 93.9 per cent.

The vaccine is a suspension of heat-killed *Mycobacterium paratuberculosis* in light mineral oil. The dose is 1 ml. of vaccine containing 5 mg. of dried bacteria. Only one injection is given and it is administered subcutaneously.

About 450,000 sheep have been vaccinated with this new type of vaccine during the past seven years. Excellent protection has been obtained.

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Polyarthritis in Sheep. I. Description of the Disease and Experimental Transmission

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A DISEASE in sheep, characterized by arthritis, has occurred in Wisconsin—both in 1957 and in 1958. On bacteriologic examination of materials from the affected articulations, microorganisms were not grown in artificial mediums or recognized on the usual microscopic examination.

The condition was transmissible and evidently caused by an organism related to the psittacosis-lymphogranuloma group of viruses. The disease is described and transmission experiments are reported in this paper. Characterization of the causative agent will be reported in a second paper [4] and serological identification in a third.[6]

NATURAL DISEASE

In both instances, the disease occurred in feeder lambs obtained in October in the western part of the United States. After an insidious onset, both the morbidity and the mortality reached their highest levels about a month after the lambs arrived in Wisconsin, with losses heaviest in late November and early December. The mortality was 3 per cent in 1957 and 7 per cent in 1958. Since both affected flocks were sent to slaughter soon after signs of acute disease subsided, the final effects are not known.

Clinical Signs.—Lameness was a prominent sign, with most of the affected lambs lame in only one leg. They carried the affected member in abduction, often dragging the hoof. When both front legs were affected, the lambs attempted to run on their hindlegs when frightened. When both hindlegs were affected, they stood in a crouched position and, when forced to move quickly, had difficulty controlling the affected legs, or they lifted them simultaneously. Pain may have accounted in part or entirely for the peculiar rigidity of the affected legs. Sometimes lambs with all four legs involved would fall in tetanus-like spasm when attempting to move. The proximal articulations were always more severely affected. Mild lameness sometimes disappeared after a few steps only to reappear after a short rest. Some apparently recovered animals developed sudden acute lameness that usually subsided completely in a few hours.

Depression was invariably associated with the disease, even in lambs that were not lame. The respiratory rate was increased. The body temperature ranged from normal to 106 F. The greatest number of clinically ill animals and mortalities occurred in a period of one to two weeks. Toward the end of the second week, most of the affected lambs improved markedly and eventually recovered; a few were permanently crippled. The appetite was completely lost in only the most severely affected. Weight loss, which was often severe, was not readily regained upon recovery.

Necropsy Findings.—A most significant and constant necropsy finding was a serous to serofibrinous or fibrinous synovitis (fig. 1). Where no fibrin was present in obviously painful articulations, the fluid varied in amount from normal to excessive and usually appeared normal except for being thin or watery. Excessive accumulation of serous exudate was frequently associated with enlargement, extensive periarticular edema, and numerous pin-point hemorrhages in the soft joint tissues and regional muscles. Articulations containing little fluid and much fibrin were in contrast to those with a serous synovitis. Such fibrin was often in a fairly compact greenish gray mass which almost completely

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filled the articular space. These masses were sometimes composed of several pieces grouped together and were either loose or attached by strands of fibrin to the synovial membrane from which they could be peeled away. These synovial membranes were dull and covered in places with fibrinous strands. Microscopic examination revealed no changes in the articular cartilages and adjacent bones.

Except for occasional areas of lung consolidation in a few lambs, no other gross pathologic changes were recognized. Investigations failed to relate the lung processes with the arthritis.

Histopathologic Findings.—Smears and impression preparations of the arthritic fluid and of synovial membranes stained with Giemsa's or Macchiavello's stain revealed, in most cases, large numbers of inflammatory cells and abnormally large, rounded synovial cells. Numerous minute coccoid bodies, which stained bright red with the Macchiavello technique and purple-red with the Giemsa stain, were also observed (fig. 2). These bodies could readily be overlooked or misinterpreted as bits of detritus or coagulated protein. They were sometimes present in large numbers in the fluid as well as in the synovial and inflammatory cells. They were designated as elementary bodies.

Sections of the arthritic joints revealed that the fibrous layer of the synovial membrane was edematous and lining cells were swollen—some greatly enlarged and at various stages of degeneration with resulting detachment, which was especially evident in the most superficial layer. The damaged synovial lining was covered with adherent strands of fibrin and inflammatory cells. The intra-articular fibrin casts contained a great number of cellular elements and debris derived from the synovia. Elementary bodies were seen frequently in large numbers in the exudate clinging to the lining membrane as well as in the "articular casts" and at the sites of advanced cellular destruction. There was evidence that these free bodies came from ruptured cells. In the affected synovial cells, the bodies were seen either loosely arranged, filling the cytoplasm almost entirely or, more frequently, as individual masses of bodies within the cytoplasm. These appeared as granular, tightly packed, large circular bodies, occasionally surrounded with a clear zone. Wherever the elementary bodies were present in a tissue, the part was usually densely infiltrated with mononuclear cells, some of which contained elementary bodies.

There were some degenerative changes in the parenchyma of the liver and the inflammatory process in the lung. Otherwise, the internal organs appeared essentially normal.

Experimental Transmission

Materials and Methods.—Lambs 3 to 8 months old were obtained from two local flocks, believed not to be affected with arthritis, for attempted transmission trials. These lambs were observed daily for two weeks before exposure and body temperatures were taken daily the last week.

Fig. 1—Opened femoropatellar joint of a sheep with polyarthritis. Fibrinous casts are visible at the tips of the wooden applicators.

Material for experimental transmission originated from 1957 cases. Fluids from affected joints that were apparently free of bacteria were inoculated by various routes into embryonating hens' eggs in different stages of incubation. Deaths occurred most consistently in embryos inoculated into the yolk sac on the sixth day of incubation.

Yolk sacs, embryonic membranes, yolk material, and extra-embryonic fluids from the second yolk sac passage were harvested individually and stored at 4 C. Those which were free of bacteria, were

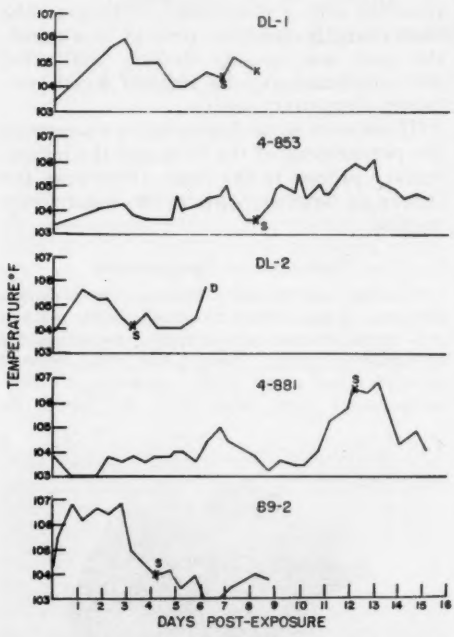

Fig. 3—Temperature response of 5 sheep experimentally exposed to second egg passage (sheep DL-1, 4-853, and DL-2) and 11th egg passage (sheep 89-2) of sheep arthritis virus, and to defibrinated blood from a naturally affected lamb (sheep 4-881). The time of appearance of clinical signs is indicated by S. Death or killing of the sheep is indicated by the letters D and K, respectively.

ground in a TenBroeck tissue grinder, pooled, and stored in equal aliquots at −30 C. Four months later, this egg material was used for the inoculation of 4 lambs (table 1). Another lamb (No. 4-881) was inoculated with defibrinated blood from a naturally infected lamb which had a temperature of 106 F. when the blood was taken. Still later, 3 lambs were inoculated with embryo material from the eleventh embryo passage. One control lamb was inoculated with material from a normal chicken embryo (not included in table 1).

When embryo material was employed as the inoculum, it was diluted equal parts with physiologic salt solution or tryptose broth and then freed of visible suspended particles through centrifugation and filtration through coarse filters (filter paper and Seitz clarifying pads 05). Ten thousand units of penicillin and 25 mg. of dihydrostreptomycin were added per milliliter of inoculum.

The inoculated animals were observed and body temperatures taken daily until they were necropsied or the signs of disease subsided. Those that made a clinical recovery were placed in a small pasture, under surveillance for the following three months. Serums were obtained and stored for future serologic tests. The animals that died or were killed when exhibiting arthritis were necropsied and visible pathological changes recorded. Tissues from the affected joints and various internal organs were fixed, either in 10 per cent buffered formalin or in Bouin's solution, sectioned, and stained with haematoxylin eosin or Giemsa stains. Smears were stained by Macchiavello or Giemsa techniques.

RESULTS

The results are summarized (table 1). Lambs DL-2 and DL-1 were severely lame, depressed, and had respiratory distress on postinoculation days 4 and 8, respectively.

TABLE 1—Polyarthritis in Sheep—Summary of Transmission Trials

Lamb No.	Exposure	Age mo.	Route	Volume in cc.	Inoculum	Response
284-2	First	8	i.p. sc.	5	2nd egg passage	Slightly lame on hind left leg on postinoculation days 6 and 7. Died 3 weeks later.
DL-1	First	3	sc. i.v.	3 5	2nd egg passage	Acutely stiff and lame on both hind legs on day 8. Markedly depressed, with respiratory distress. Killed 24 hours later.
4-853	First	3	i.v.	3	2nd egg passage	Lame on right hind leg and depressed from day 9 to 16.
DL-2	First	3	i.v.	4	2nd egg passage	Acutely stiff and lame on all legs after day 4. Depressed with accelerated breathing. Died on day 7.
4-881	First	3	i.v.	10	Defibrinated blood	Unwilling to move, depressed, fast breathing from day 12 to 14.
89-2	First	3	i.v.	5	11th egg passage	Markedly stiff and lame on both hindlegs, also depressed and fast breathing from day 5 to 9.
4-853	Second	6	i.v.	5	11th egg passage	No response.
4-881	Second	6	i.v.	5	11th egg passage	Mild lameness.

i.p. = intraperitoneal; sc. = subcutaneous; i.v. = intravenous.

Lamb DL-1 was killed while moribund 24 hours after the clinical signs were first noticed. Lamb DL-2 died three days after the appearance of lameness. Lamb 248-2 became lame on day 6, made an apparent recovery two days later, but died three weeks later with extensive bacterial peritonitis. Lamb 4-853 was lame and depressed on day 9 but recovered within a week. Lamb 4-881, which was inoculated with defibrinated blood from a sheep with natural arthritis, did not develop lameness but showed a sharp rise in temperature on day 12 (fig. 3) accompanied by depression and labored breathing.

Lamb 89-2 became lame, depressed, and dyspneic five days after inoculation with the 11th egg passage material. Two animals which had been exposed previously were also inoculated with the 11th egg passage material. Of these, lamb 4-853, which had recovered from the lameness induced by the first exposure, remained healthy; lamb 4-881, which had developed a temperature reaction but no lameness at the time of the first exposure, showed mild lameness eight and nine days after the second inoculation but no generalized response.

The clinical manifestations of the experimental disease were identical to those observed in natural cases. Gross pathologic changes observed in the lambs DL-1 and DL-2 were polysynovitis, of sero-exudative type, involving primarily the elbow, stifle, and hock joints. There was an excess of grayish, watery joint fluid; also numerous small hemorrhages in the synovial membranes in the periarticular soft tissues, and in the round ligaments of the hip joints. Areas of consolidation were observed in the lungs of animal DL-1. Scattered small hemorrhages on the epicardium and in the lungs, but no areas of consolidation, were noticed in animal DL-2. A slight excess

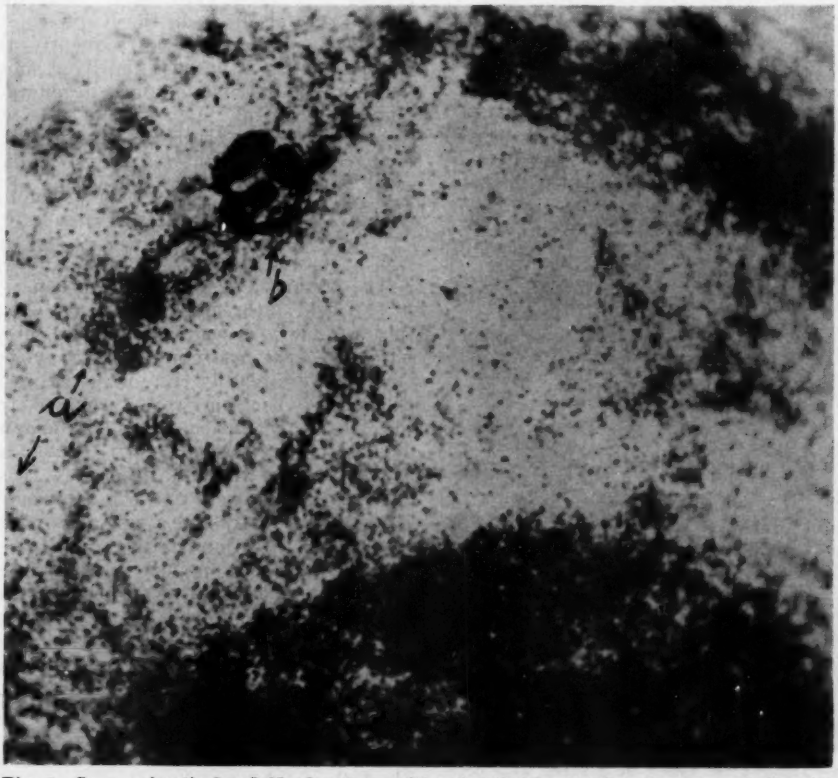

Fig. 2—Smear of articular fluid of a sheep with polyarthritis, showing elementary bodies (a) and inflammatory cells (b). Giemsa's stain; x 1,400.

of pericardial, pleural and peritoneal fluid may have been present. Otherwise the internal organs appeared grossly normal.

Direct microscopic examination of the smears made from the joints and the various body fluids were negative for bacteria. Cultures of various internal organs and of the body fluids yielded only coliform organisms from the lungs of lamb DL-1 and *beta*-hemolytic staphylococci from the lungs and spleen of lamb DL-2.

Smears of the joint fluid consistently contained many inflammatory and synovial cells. Pathologic changes in cross sections of the synovial membranes were essentially the same as those described in natural cases. Cellular inclusions with elementary bodies were observed both in smears and tissue sections of the synovial membranes. On occasion, elementary bodies were found in great numbers in the synovial exudate.

Changes revealed on examination of sections of the pneumonic areas of lamb DL-1 indicated bacterial infection. Other areas of the lung located more peripherally and not in immediate contact with the pneumonic parts were affected with an interstitial pneumonia with thickened interalveolar tissue. Such thickening resulted from edema and an increase in size and numbers of interstitial elements. Comparable changes were found in the lung sections of lamb DL-2, which did not have gross abnormalities of the lungs, except for small hemorrhages. Involvement of the alveolar epithelium was apparent. Thickening of the visceral pleura and peritoneum, which did not seem to be the result of shrinkage, was observed. Occasional interstitial or mononuclear cells harboring elementary bodies were seen in the interstitial and subserosal tissues.

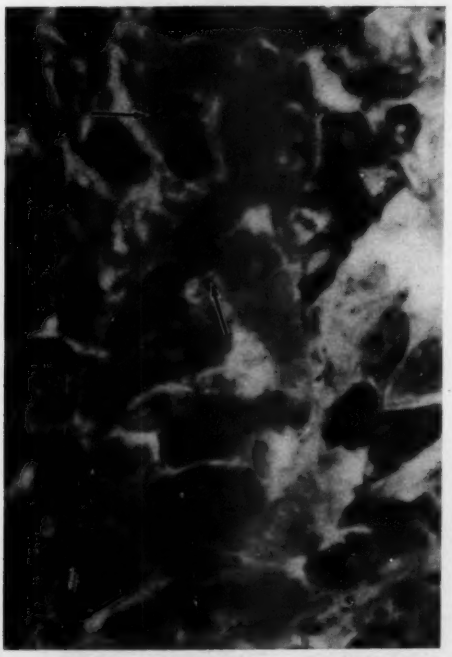

Fig. 4—Tissue from spleen of a sheep with polyarthritis. Arrows point at cells containing elementary bodies. Giemsa stain; x 1,300.

Elementary bodies were also found in the spleen (fig. 4), liver, and kidney in decreasing order of frequency. The most striking change in the spleen was the hyperplasia of the reticular cells. It was not unusual to find several cells in these areas with large numbers of elementary bodies engulfed in their cytoplasm. Elementary bodies were also seen in the Kupffer's cells of the liver though less frequently than in the spleen. Otherwise the liver showed widespread parenchymatous degenerative changes, central edema, and scattered hemorrhages. Similar degenerative changes were encountered in the kidneys, especially in the tubular epithelium of the medullary zone.

DISCUSSION

The arthritic condition of sheep, seen in two field outbreaks, is believed to represent a newly recognized disease entity. *Erysipelothrix rhusiopathiae* and pyogenic bacteria were readily eliminated as possible causes. No bacteria were recovered from the joints of affected sheep. Pleuropneumonia-like organisms (PPLO) have been found to cause arthritis in sheep.[5] In several trials, PPLO were not isolated from the joints of naturally and artificially infected sheep. The infectious nature of the disease described in this paper was apparently proved by successful experimental transmission using material from the second and 11th chicken embryo passage of the joint fluid from a naturally affected lamb. The experimental disease was strikingly similar to the natural one, both clinically and pathologically.

The identity of the etiologic agent was not fully established but there were many indications that it belongs to the psittacosis-

lymphogranuloma venereum group of viruses. These indications include the finding of small coccoid bodies, stained red with Macchiavello and purple-red with Giemsa stains, in the joints of naturally and artificially infected sheep, as well as in some of the visceral organs; also the finding of the same bodies in chicken embryos inoculated with material from the joints of affected sheep. (Later findings also indicate that the causative organism belongs to psittacosis-lymphogranuloma group.[4]).

Recovery from sheep arthritis was apparently followed by immunity in lamb 4-853 (table (1). This lamb developed arthritis after the first exposure, but remained healthy following re-exposure three months later. However, lamb 4-881 seemed to be only partially immune, since it developed mild lameness after re-exposure.

The elementary bodies were seen principally in the joints, but were also found in lung, spleen, liver, and kidney of the experimentally infected animals. The mode of spread of the virus from sheep to sheep is unknown. In one instance, in which the agent must have survived for some time outside the body, two groups of healthy feeder lambs developed arthritis when introduced into the premises one and two weeks after the infected flock was sent to slaughter. There was serologic evidence that they were affected with the arthritis virus.[4]

Viruses of the psittacosis-lymphogranuloma group are known to cause infectious diseases in sheep (ovine abortion[7] and lamb pneumonia[1,2]) and in other domesticated animals.[3,8] The arthritic condition reported here appears to be clinically and pathologically different from both ovine abortion and lamb pneumonitis. Whether the virus of sheep arthritis is also different from the agents of the other two diseases of sheep is now being investigated.[6]

SUMMARY

An apparently new disease, polyarthritis of sheep, occurred in Wisconsin in the fall of 1957 and again in 1958. A virus-like agent was recovered from the joints of naturally infected lambs and propagated serially in chicken embryos. Susceptible lambs inoculated with the chicken embryo-propagated virus developed a condition indistinguishable from the disease seen in the field. The etiologic agent seemed to belong to the psittacosis-lymphogranuloma group of viruses.

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Polyarthritis in Sheep. II. Characterization of the Causative Virus

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THE CLINICAL and pathologic features observed in sheep with infectious polyarthritis were described in a preceding paper.[4] An agent was isolated, in chicken embryos, from naturally infected sheep, and the disease was experimentally reproduced. Further investigations of the characteristics of the agent, its pathogenicity for laboratory animals, and its serological behavior established its relationship with the psittacosis-lymphogranuloma group of viruses. The result of these investigations form the basis for the present report.

MATERIALS AND METHODS

The virus was passed serially in 6-day-old embryonating chicken eggs by the yolk sac route. The inoculum consisted of 0.2 ml. of arthritic fluid or of yolk sac membrane from infected embryos suspended in the embryonic fluids. The inoculated eggs were candled twice daily and the egg yolks were harvested soon after death of the embryos.

Guinea pigs ranging in age from 2 to 12 months were inoculated intraperitoneally with 0.5 ml. of infected egg material or articular fluid from affected sheep.

Young adult Swiss mice were inoculated intraperitoneally, intravenously, or subcutaneously with 0.2 ml., and intranasally with 0.05 ml. of infected egg suspension. Suckling mice, 17 days old, were inoculated intracerebrally with 0.02 ml. of the same material, using at least 5 mice for each type of inoculation. The mice were kept under observation three to six weeks after inoculation.

One rabbit was given 10 intravenous inoculations (1 ml.) on alternate days. One week after the last inoculation, the rabbit was exsanguinated and the blood serum recovered.

A number of serum samples from sheep with natural and experimental arthritis were tested for complement-fixing antibodies using both ornithosis* and sheeep arthritis viral antigens.

Homologous complement-fixing antigens were prepared from yolk sacs infected with sheep arthritis virus according to the procedure of Whitney and Gnesh.[5] Only eggs with numerous elementary bodies in yolk sac smears were selected for the preparation of CF antigen. No anticomplementary activity was noticed. Complement-fixation tests were performed following the procedure of Bedson *et al.*[2] Two hemolytic doses of complement and 5 hemolytic doses of hemolysin were used. Overnight fixation at 4 C. was followed by 30 minutes' incubation at 37 C. with the hemolytic system, after which the reaction was read. The tubes were refrigerated for two hours at 4 C., and a second reading was taken. The percentage of hemolysis was estimated by visual inspection and the reactions graded as follows:

++++ = no hemolysis;
+++ = 25 per cent hemolysis;
++ = 50 per cent hemolysis;
+ = 75 per cent hemolysis;
negative = 100 per cent hemolysis.

Only tubes with a ++ reaction or greater were considered positive.

RESULTS

Pathogenicity of the Virus for Chicken Embryos and Laboratory Animals.—Inoculation of chicken embryos with the original articular fluid always resulted in embryonic death in five to 14 days after inoculation. After a few serial passages, however, death occurred invariably in four days after inoculation. The chicken embryos showed hemorrhages, but no other gross changes. Most of the embryos inoculated by routes other than the yolk sac survived; the few that died were stunted, curled, and had deformed joints. Some of these embryos had necrotic foci in the

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* The complement-fixation tests with ornithosis antigen were performed by Dr. H. Brumfield, Division of Veterinary Bacteriology and Public Health, College of Veterinary Medicine, University of Minnesota, St. Paul.

liver and a fibrinous exudate on the surface of internal organs. The same lesions occurred in some of the chicken embryos that had been inoculated into the yolk sac with small amounts of virus, and had died eight to 15 days later.

Elementary bodies were found in smears of the yolk sac, yolk, amniotic fluid and, in small numbers, in the allantoic fluid of the infected chicken embryos.

Infectivity titers calculated with the formula of Reed and Muench [7] were between 5×10^7 and 5×10^8, 50 per cent chicken embryo lethal doses (e.l.d.$_{50}$) per milliliter. Survival time increased from 4 days in eggs inoculated with undiluted material, to 15 days in eggs inoculated with dilutions of 10^{-8}. This survival time pattern was consistent in several replicate experiments (table 1).

TABLE 1—Average Death Time of Chicken Embryos Inoculated Via the Yolk Sac with Tenfold Dilutions of Infectious Egg Material

Dilution	No. embryos dead/ No. inoculated	Av. days postinoculation until death
Undiluted	6/6	4
10^{-1}	6/6	6
10^{-2}	6/6	6
10^{-3}	6/6	7
10^{-4}	6/6	8
10^{-5}	6/6	9
10^{-6}	6/6	11
10^{-7}	5/6	12
10^{-8}	3/6	15
10^{-9}	0/6	....

Some indication of the relative concentration of virus in the various fluids of the chicken embryo and in the yolk sac membrane was obtained by comparing the survival times of embryos inoculated via the yolk sac with different materials (table 2).[3] The same survival time (6 days) was observed in embryos inoculated with undiluted amniotic fluid and with yolk sac membrane or yolk diluted 10^{-2}. Embryos inoculated with undiluted allantoic fluid died three days later than those inoculated with amniotic fluid or with a 10^{-2} dilution of yolk sac or yolk.

Guinea pigs were susceptible to the sheep arthritis virus. All of the 19 inoculated guinea pigs died or were killed when moribund. Deaths occurred from four to nine days after inoculation. The clinical signs included anorexia, depression, marked loss of body weight, and labored breathing with marked respiratory râles. Reluctance to move and bleeding from the rectum were occasionally observed. Serous to serofibrinous pleuritis and peritonitis were constantly observed at necropsy (fig. 1). The fibrinous exudate was easily peeled off the underlying organs, which were friable and congested. Enteritis occurred frequently.

TABLE 2—Average Death Time of Chicken Embryos Inoculated Via the Yolk Sac with Different Materials from Infected Chicken Eggs

Inoculum		Av. days postinoculation until death	Probable* e.l.d.$_{50}$/ml.
Material	Dilution		
Yolk sac membrane	10^{-2}	6	$5 \times 10^{6.35}$
Yolk	10^{-2}	6	$5 \times 10^{6.35}$
Amniotic fluid	Undiluted	6	$5 \times 10^{6.35}$
Allantoic fluid	Undiluted	9	$5 \times 10^{3.35}$
Allantoic fluid	10^{-2}	11	$5 \times 10^{2.35}$

* The probable infectivity titers in e.l.d.$_{50}$ are based on the average death time.[3]

Elementary bodies were seen in great numbers in smears of the serofibrinous exudate of infected guinea pigs. Elementary bodies were also found in the cyto-

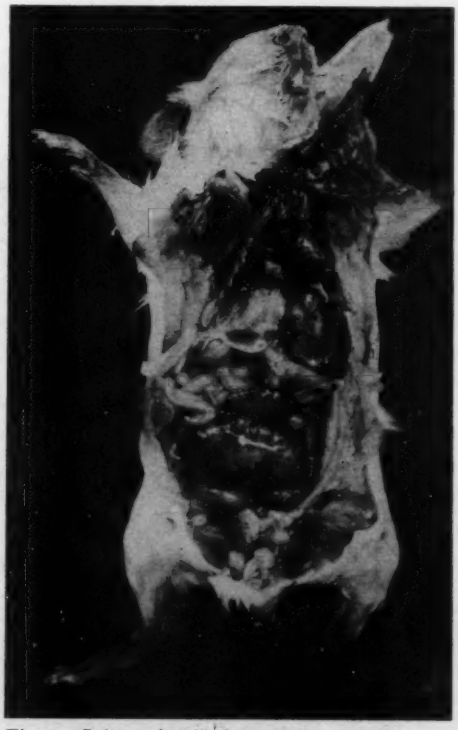

Fig. 1—Guinea pig killed two days after exposure to sheep arthritis virus. Fibrinous exudate is visible on the peritoneum, especially on the surface of the liver and spleen.

TABLE 3—Effect of Antibiotic Treatment of Sheep Arthritis Virus on the Average Death Time of Inoculated Chicken Embryos

Antibiotic	Concentration per ml. inoculum	Delay in av. death time	Probable reduction* in titer (e.l.d.$_{50}$/ml.)
Penicillin	10 units	No delay	0
Penicillin	100 units	6 days	10^5
Penicillin	1,000 units	8 days	10^7
Streptomycin	200 mgr.	No delay	0

* The probable reduction in titer by antibiotic treatment is based on the average death time.[3]

plasm of many reticuloendothelial cells in sections of spleen, liver, and lung of infected guinea pigs. Plaques or large bodies were seen in the mesothelial cells of the serosal membranes (fig. 2, 3). Extensive cell proliferation and necrosis, exudation and hemorrhages were seen in those tissues where elementary bodies were present. The histologic picture was similar to that described for experimentally infected sheep;[4] however, there was a greater degree of degenerative changes.

Young adult and suckling Swiss mice failed to respond to inoculation of infected egg material. No pathologic change was observed in mice at necropsy. One rabbit withstood repeated intravenous inoculations of infected egg material without apparent illness.

Effect of Antibiotics, Formalin and Temperature.—When 100 units of penicillin or more was added, per milliliter of inoculum, mortality of the chicken embryos was delayed (table 3). When 10,000 units of penicillin/ml. of inoculum was added, the embryos survived until hatching time, but then either died or were too weak to break through the eggshell. Hatching time was often delayed. Infectious virus was recovered from the yolk sac membranes of these embryos. Streptomycin in concentrations, up to 200 mg./ml. of inoculum, appeared to have no effect on the death pattern of chicken embryos.

When 4 infected guinea pigs were inoculated intraperitoneally with 10 mg. of terramycin daily, beginning 24 hours after exposure, and with 100 mg. daily from the time clinical signs of illness were noticed, the course of the disease was not influenced.

Four healthy guinea pigs were given a series of subcutaneous inoculations of infected guinea pig tissue suspension containing 0.5 per cent formalin. Eleven days after the last subcutaneous inoculation, they were injected intraperitoneally with

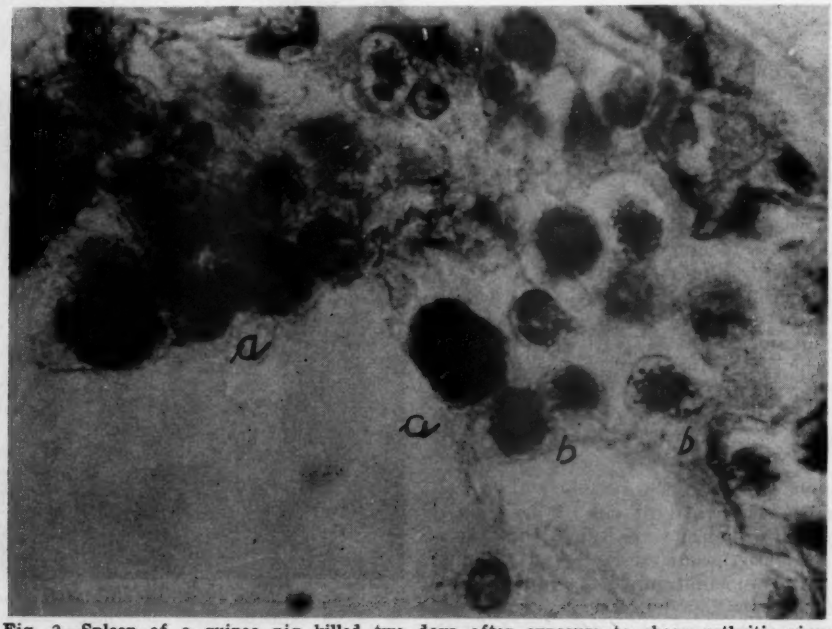

Fig. 2—Spleen of a guinea pig killed two days after exposure to sheep arthritis virus. Large, homogeneous inclusion bodies (a) and intracellular elementary bodies (b) are clearly visible. Giemsa's stain; x 1,300.

the same guinea pig tissue suspension without formalin. Of the 4 guinea pigs, 3 died eight to 12 days later; the fourth survived.

The infectivity titer of a preparation of fresh egg material was $5 \times 10^{7.84}$ e.l.d.$_{50}$/ml. After freezing and thawing, the titer was $5 \times 10^{6.62}$. At room temperature, there was no loss of titer in infectious egg material after one week but after two weeks the titer dropped by four logs, after 16 days by seven logs, and after three weeks no infective virus was recovered. Incubation of infectious egg suspension at 37 C. for 15 hours did not affect the infectivity titer.

Virus Neutralization and Complement Fixation.—Either 100 or 1,000 e.l.d.$_{50}$ of virus were mixed with serial dilutions of serum from experimentally or naturally infected sheep. The serum-virus mixtures were then inoculated into egg embryos by the yolk sac route. In no case did the inoculated embryos survive. However, death was delayed in those given the greater concentrations of serum. Normal sheep serum did not delay embryonic death. No correlation was observed between virus-neutralization and complement-fixation titers.

A number of serum samples from lambs with naturally occurring arthritis were used in complement-fixation (CF) tests with ornithosis antigen and with antigen prepared from sheep arthritis virus (table 4). Fifteen of 20 serums had titers ranging from 1:8 to 1:64 with ornithosis antigen; five serums were negative. Two lambs (J-1 and J-2) had a CF titer of 1:32 at the time of illness, but became negative on CF within 16 days. Twelve serums tested with sheep arthritis antigen had titers ranging from 1:8 to 1:64.

Serums collected at different times from experimentally infected lambs were also tested by complement-fixation (table 5). Since the experimental sheep had been exposed to infected yolk sac, "antigen" prepared from uninoculated yolk sac was included in this test. The CF titers ranged from 1:8 to 1:256 with ornithosis antigen, from 1:32 to 1:64 with sheep arthritis antigen, and from 1:4 to 1:16 with normal

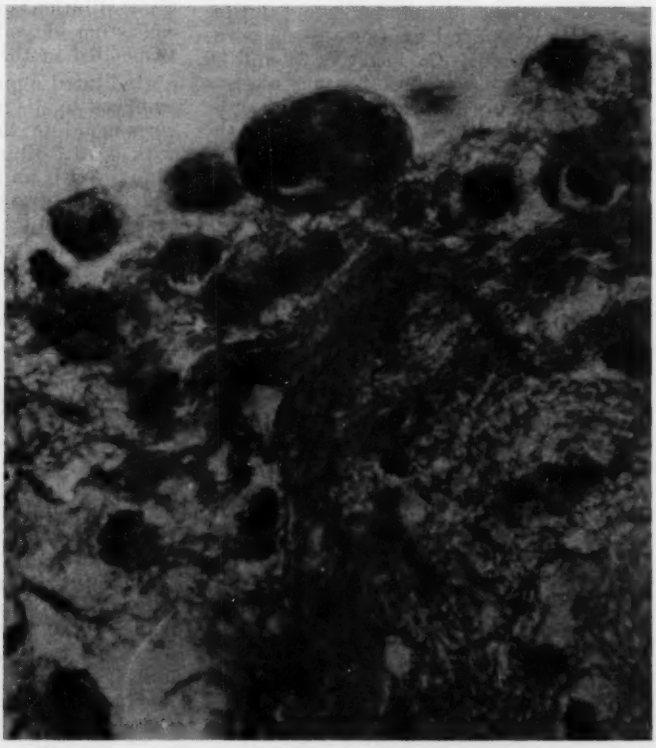

Fig. 3—Same as figure 2. Two plaques are visible within one greatly enlarged cell. Elementary bodies can be seen in one of the plaques. Giemsa's stain; x 1,400.

TABLE 4—Complement-Fixation (CF) Titers of Serums from Sheep Affected with Arthritis

Sheep No.	CF Antigen: Ornithosis	CF Antigen: Sheep arthritis	Remarks
J-8	1:64	1:64	.....
J-4	1:64	1:64	.....
9347	1:64	1:32	.....
J-3	1:64	1:32	Very lame. Virus isolated.
J-15	1:64	1:32	.....
J-1	1:32	1:64	.....
J-1	1:16	.....	Second blood sample, 9 days after first.
J-1	Negative	.....	Third blood sample, 16 days after first.
J-5	1:32	1:32	.....
J-13	1:32	1:32	.....
J-2	1:32	1:16	.....
J-2	Negative	.....	Second blood sample, 9 days after first.
J-2	Negative	.....	Third blood sample, 16 days after first.
J-14	1:16	.....	.....
			.....
J-6	1:8	.....	.....
J-9	1:8	.....	.....
J-11	1:8	.....	.....
J-12	1:8	.....	.....
J-7	Negative	.....	.....
J-10	Negative	.....	.....
J-16	.....	1:64	.....
J-18	.....	1:64	.....
J-17	.....	1:8	.....

yolk sac antigen. Lamb 4-853 had a titer of 1:8, 17 days after exposure to the virus of sheep arthritis, was negative 27 and 60 days after exposure, but became positive after re-exposure to the virus. No complement-fixing antibodies were detected in the serum of infected guinea pigs, with one exception; this was 1 of 4 guinea pigs that had been vaccinated with formalin-treated virus of guinea pig origin and subsequently challenged with virulent guinea pig material. The serum of this animal collected at the time of death, 12 days after challenge with virulent virus, had a CF titer of 1:32 with ornithosis antigen.

TABLE 5—Complement-Fixation (CF) Titers of Serums from Sheep Experimentally Exposed to Sheep Arthritis Virus

Sheep No.	Days after exposure (I) or re-exposure (II)	CF Antigen: Ornithosis	CF Antigen: Sheep arthritis	CF Antigen: Normal yolk
4-853	17(I)	1:8	.....	.....
4-853	27(I)	Negative	.....	.....
4-853	60(I)	Negative	.....	.....
4-853	8(II)	1:16	.....	.....
4-853	14(II)	1:32	1:64	1:16
4-853	28(II)	1:16	.....	.....
4-853	42(II)	1:8	.....	.....
89-2	8(I)	1:256	1:64	1:16
89-2	14(I)	1:64	1:64	1:16
89-2	28(I)	1:32	1:32	1:16
89-2	42(I)	1:64	1:32	1:4
4-881	20(II)	1:32	1:32	1:8

DISCUSSION

Inoculation of 6-day-old chicken embryos via the yolk sac was the most convenient method of propagating the virus of sheep arthritis. This is also the preferred method for propagation of viruses of the psittacosis-lymphogranuloma group. The agent of sheep arthritis reached high titers in the yolk sac of inoculated eggs. When various fluids from infected chicken embryos were used as inoculums, the survival time of the inoculated embryos gave some measure of the relative concentration of virus in the embryonic fluids.

Golub [3] showed that a linear relationship exists between the concentration of viruses of the psittacosis-lymphogranuloma group inoculated into eggs by the yolk sac route, and the average death time of the embryos. He stated that it is possible to estimate directly the e.l.d.$_{50}$ value of a virus suspension from the average death time of a group of eggs inoculated with one dilution. A 10^{-2} dilution of yolk sac or yolk caused embryonic death in 6 days (table 2). This was the survivial time for the same dilution of the standard inoculum consisting of yolk and yolk sac membrane suspended in the embryonic fluids.

Undiluted amniotic fluid also caused the embryos to die in six days, which would correspond to the survival time obtained with a 10^{-2} dilution of the standard inoculum. With undiluted allantoic fluid, the survival time was nine days, the same as with a 10^{-5} dilution of standard inoculum. These results indicated that the virus was in greatest concentration in the yolk membrane and yolk, followed by the amniotic and allantoic fluids in that order. The same impression was gained by examination of smears of these various materials and visual estimation of the numbers of elementary bodies.

The virus of sheep arthritis was pathogenic for guinea pigs. The lesions observed in guinea pigs were those resulting from exudative inflammatory processes of all the serous membranes. Generalized infection occurred in the guinea pigs, as judged by signs of depression and body atrophy and by the distribution of elementary bodies throughout the visceral organs. In attempts to control the infection in 4 guinea pigs by administration of terramycin, the course of the illness was apparently prolonged but death eventually oc-

curred. Attempts to produce immunity in 4 guinea pigs, by subcutaneous inoculations with formalin-treated virus, met with limited success; the survival time after challenge with virulent virus was somewhat prolonged in 3; however, the fourth animal survived.

With one exception, CF antibodies were not detected in the serum of infected guinea pigs. This may have been due to the rapid course of the disease which may not have allowed enough time for antibody formation. One may also take the contrary view, that the insufficient antibody formation accounted for the rapidly fatal disease.

Suckling and young adult Swiss mice were resistant to exposure by a number of different routes. This appears to be definitely different from the reported susceptibility of mice to viruses of the psittacosis-lymphogranuloma group;[4] in this group, only the agent of sporadic bovine encephalomyelitis is not pathogenic for mice.[5]

The agent of sheep arthritis was somewhat sensitive to penicillin and terramycin, but not to streptomycin. This is in agreement with the reports on antibiotic sensitivity of psittacosis-lymphogranuloma viruses.[5]

Although convalescent sheep serum did not prevent death of chicken embryos inoculated with 100 and 1,000 e.l.d.$_{50}$ of virus, some protection was obtained in that embryonic death was delayed; this did not occur with normal serum. The results of the viral neutralization tests were not clear enough to make this procedure useful in the diagnosis of the disease.

Satisfactory CF antigens were prepared by lyophilization of suspensions of infected yolk sacs. The antigenic potency, although not extremely great, was sufficient to obtain titers comparable to those given by an ornithosis antigen with the same serums.

An interesting finding was that phenol destroyed the activity of the CF antigen. Phenol is known to destroy the strain-specific antigen of viruses of the psittacosis-lymphogranuloma group, but it leaves unaltered or enhances the activity of the group specific antigen.[1] No residual CF activity was found in the antigenic preparations of sheep arthritis virus after phenol treatment. It would seem, therefore, that the group antigen was destroyed during the preparation and lyophilization of the yolk sac suspensions, although the agents reportedly capable of destroying the group antigen (KIO_4, dilute HCl) were not employed. Tests with antiserums to other viruses of the psittacosis-lymphogranuloma group should reveal whether the CF antigen used in this work possesses group or type specificity.

The serum of most sheep with arthritis contained CF antibodies for both ornithosis and homologous antigens but these antibodies did not persist long in 2 naturally affected sheep. Sheep J-1 had a titer of 1:32 with ornithosis antigen at the time of lameness, but nine days later the titer was 1:16, and 16 days after the first bleeding the CF reaction was negative. Sheep J-2 also had a titer of 1:32 when showing signs of illness, but no CF reaction was detected nine and 16 days after the first blood sample was taken (table 4). One experimentally exposed lamb (4-853, table 5) had a similar response, with a titer of 1:8 on postexposure day 17, but no reaction on days 27 and 60. These antibodies persisted in the serum of another experimentally infected lamb (89-2, table 5) for at least 42 days after exposure. Experimental lambs had CF antibodies to normal yolk sac antigens; however, the titers were lower than those to sheep arthritis antigen in the same serums (table 5).

CONCLUSIONS

The virus of sheep arthritis seems to be related to the psittacosis-lymphogranuloma group. Similarities with viruses of this group include: the presence of elementary bodies, relative sensitivity to terramycin and penicillin but not to streptomycin, pathogenicity for chicken embryos and guinea pigs and, more important, cross reaction with ornithosis CF antigen. Some differences were observed, notably the resistance of mice to experimental infection and destruction of the CF antigen by phenol.

More information is needed to decide whether sheep arthritis is caused by a new virus of the psittacosis-lymphogranuloma group, or whether it is a hitherto undescribed manifestation of an already known virus.

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The Effects of Light and Temperature on Ovarian Activity in Sheep

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IN GENERAL, the breeds of sheep common in North America exhibit estrus during the fall months and are best described as seasonally polyestrous breeders. However, some sheep are less seasonal in breeding behavior than others, particularly those of Merino, Rambouillet, and Dorset origin.

Following Marshall's[8] observation that sheep and deer transported from one hemisphere to another underwent a reversal of the breeding season, it was hypothesized that the initiation of the breeding season in sheep is associated with a reduction in day length. This hypothesis was substantially verified by Sykes and Cole,[11] Yeates,[12] Hart,[5] and others.

Hafez[6] concluded that there is a relationship between the onset and length of the breeding season and the latitude and altitude of the native home of six breeds of British sheep. Breeds from the Scottish Highlands exhibit estrus when day length is reduced to 11.5 to 12.5 hours and the breeding season lasts for about 19 weeks. Dorset sheep, which presumably are derived in part from the Spanish Merino, come into estrus when day length is 12 to 17 hours or less, and have a breeding season of about 32 weeks.

Under natural conditions, a reduction in day length is accompanied by some reduction in ambient temperature, and the possible effects of temperature alone or temperature-light interactions cannot be ignored. Phillips and McKenzie,[10] McKenzie and Berliner,[9] Gunn *et al.*,[4] and others have shown that high ambient temperatures are associated with reduced semen quality and fertility of rams. It has since been shown that ewes maintained under natural light conditions at 45 to 48 F., from May 26 to October 8, came in heat eight weeks earlier than ewes kept in a room with similar natural light but without cooling (Dutt and Bush[1]).

The object of this study was to determine the effects of a reduction in day length on ovarian activity.

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MATERIALS AND METHODS

Three groups of 10 yearling western ewes were used. They were a mixture of Rambouillet, Hampshire, and Suffolk breeding. All animals were fed the same ration of alfalfa hay, shelled yellow corn, whole oats, and protein supplement. The ewes were shorn during the first week of the experiment. The control animals, group I, were kept in an open shed under normal light and temperature conditions. The other two groups were kept in insulated, completely darkened rooms for a specified time interval each 24 hours, and during the remainder of the time were kept outside under normal light and temperature conditions. Group II was given 11 hours of natural light each day and was confined in the dark rooms from 6:30 p.m. until 7:30 a.m. Group III was subjected to a gradually reduced amount of light. The first week, the sheep were kept 13.0 hours in natural light, the second week 12.5 hours, and day length was reduced 30 minutes each week until the animals were getting 11 hours of daylight and 13 hours of darkness. The latitude of Lafayette is 40° N. and the altitude 586 ft.

The experiment was begun on April 10 and terminated on July 24. On June 19, two air-conditioning units of 1-ton refrigeration capacity each were installed in the dark rooms to prevent overheating. From this time on, the night temperature of the ewes in the dark rooms did not exceed 78 F. or fall below 65 F. During the day, however, all sheep were exposed to normal environmental temperatures.

TABLE 1—The Effect of Decreased Light on the Occurrence of Estrus

Treatment	I Control	II 11 hr. daylight	III Decreasing to 11 hr. daylight
No. ewes	10	10	10
No. ewes showing estrus	3	6	7
Range of onset of first estrus	April 11–May 6	May 3–July 4	April 12–July 10
Total estrual periods	5	10	12
No. ewes that conceived	0	1	1
Conception date	..	June 19	July 2

Two Hampshire rams were kept under normal environmental conditions throughout the experi-

ment. All ewes were checked for estrus each night and morning and were mated whenever found to be in heat. No attempt to evaluate semen quality of the rams was made.

Direct observation of the ovaries, when made, was through a ventral midline incision. All follicles 1 mm. or larger were measured with a caliper, and numbers of corpora lutea were recorded. Four ewes were operated on in this manner June 4 and 5, as a preliminary observation of the effects of treatment on ovarian function; the other ewes were similarly examined between July 16 and 22.

RESULTS AND DISCUSSION

Three of the 10 control ewes exhibited estrus during the 15-week period (table 1). One was in heat on the second day of the experiment, was mated, returned in heat 16 days later, was rebred, and showed no further evidence of estrus. At laparotomy on July 22, no corpora lutea were found and there was no evidence of pregnancy at that time. A second control ewe experienced two apparently normal heat periods and a third ewe was in heat once on May 6. None of the control ewes showed evidence of ovulation or corpus luteum formation and none lambed.

Thirteen of the 20 ewes which were subjected to an 11-hour day, either by an immediate or gradual change in day length, exhibited at least one heat period.

A relatively large number of follicles, ranging from 1 to 3 mm. in diameter, were present when the ovaries were examined by laparotomy. There was no increase in follicles of 4 to 7 mm. diameter in the ewes which were subjected to a reduced amount of light, but ovulation and corpus luteum formation were increased in the light-treated animals. The role of temperature in this response is not clear.

TABLE 2—The Effect of Decreased Light on Ovarian Activity

Treatment	I Control	II 11 hr. daylight	III Decreasing 11 hr. daylight
No. ewes	10	10	10
Total No. of follicles			
1–3 mm.	81	70	66
4–5 mm.	19	8	10
6–7 mm.	5	10	5
Total No. of corpora lutea	0	8	12

The daytime temperatures to which all sheep were subjected ranged from 54 to 94 F. The range in night temperatures in the open shed was 34 to 68 F. After June 19, when cooling was provided in the dark rooms, the night temperatures ranged between 65 and 78 F. Since the night temperatures in the dark rooms were somewhat higher than the normal night temperatures in the open shed, it would appear that any differences in response would be due to differences in light.

The presence of relatively equal numbers of follicles in the control and treated ewes was not unexpected. Kammlade *et al.*[7] had previously shown that anestrous ewes have large numbers of follicles 1 mm. or more in diameter and that the largest follicles in anestrous ewes are approximately the same size as those present during the breeding season. They hypothesized that anestrus is due to an imbalance between follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and that relatively little LH is secreted during anestrus. The principal effect of the light treatment in the present experiment was the induction of ovulation and corpus luteum formation. As reported in reviews by Yeates,[13] Hart,[5] and others, the effects of light are mediated through the anterior pituitary gland and it is reasonable to presume that changes in pituitary gonadotropic hormone relationships account for the ovarian changes.

There are distinct differences between sheep in seasonal breeding patterns. Whiteman and Brown [12] studied the fertility of 200 Rambouillet and Rambouillet x Panama-Rambouillet ewes in Oklahoma between May 20 and July 12 during two different years. The experiment involved the effects of shearing time of the ewes, and the artificial cooling of rams; with respect to seasonal effects alone, 148 of 189 early-sheared and 170 of 194 late-sheared ewes lambed. It is evident that some sheep will breed outside of the usual fall lambing season and even while day length is increasing.

Yeates [13] concluded that light is the principal regulating agent of the reproductive cycle of sheep and that with the exception of nutritional status, no other stimuli have been shown to alter the seasonal reproductive pattern.

Several recent studies, Dutt *et al.*,[2] Foote *et al.*,[3] and Whiteman *et al.*,[12] indicate that cooling does improve the fertility of rams. The effects may be the result of lowered testicular temperature, improved fer-

tilizing capacity of the sperm, or reduced embryonic mortality. These experiments verify those referred to earlier in this paper.

The fact that environmental temperature does affect the fertility of rams, and that Dutt and Bush [1] were able to induce early estrus by lowered temperature alone, indicates that much work needs to be done on all environmental factors and the interactions of the individual factors.

SUMMARY

Ten yearling western ewes were maintained in an open shed under normal environmental conditions from April 10 to July 24. Three of these control ewes had one or two estrous periods but none ovulated.

Of 20 ewes exposed to reduced amounts of natural light by confinement in a darkened room during a part of the 24-hour cycle, 13 came in heat and were bred, but only 2 produced lambs.

A reduction of the light period to 11 hours of natural light, with 13 hours of complete darkness per day beginning on April 10, increased the incidence of estrus and induced ovulation but fertility remained low.

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The Effect of Cadmium Compounds on *Strongyloides papillosus* in Sheep

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No EFFECTIVE TREATMENT has heretofore been reported against *Strongyloides papillosus*, a nematode parasite found in the small intestines of sheep and cattle. In a search for nematocidal compounds, several cadmium compounds were found to be highly active against *S. papillosus* in sheep. The present paper reports the results of studies carried out in the summer of 1958.

Cadmium oxide and other cadmium compounds are used commercially mixed with the feed in treating *Ascaris suum* infections in swine.[1-3] Cadmium oxide has also been used effectively against *Parascaris equorum* infections in horses.[4] When mixed with the feed, cadmium oxide markedly reduces the numbers of adult and larval *Trichinella spiralis* in experimentally infected mice.[5, 6]

In screening tests against horse strongyle larvae *in vitro*, Levine and Ivens[7] found that cadmium iodide was highly effective, killing all larvae at a concentration of 0.00025 M. Levine and Ivens[8] and Levine *et al.*[9] found that cadmium chloride killed all larvae at a concentration of 0.0005 M; cadmium bromide, cadmium nitrate and cadmium acetate did so at a concentration of 0.001 M; cadmium oxide, at 0.0025 M; and cadmium sulfate and cadmium anthranilate, at 0.01 M. Cadmium carbonate and cadmium sulfide were ineffective at 0.01 M.

MATERIALS AND METHODS

All animals used were grade Columbia-type lambs, with the exception of lamb 0, a Suffolk. The drugs were administered *per os* in capsules. Fecal nematode egg counts were made before treatment, three to five days after treatment, and periodically thereafter for as long as four months, using the McMaster technique[10] with Sheather's sugar solution as the flotation fluid. The lambs were weighed before treatment, three to five days after treatment, and at different intervals thereafter. They were observed for clinical signs of intoxication during the first four to five hours after treatment and periodically thereafter. Fecal egg counts and weights of untreated control lambs were determined at the same time as the treated ones.

RESULTS

The results are given (table 1). Cadmium iodide and cadmium oxide at the rate of 100 mg. per kilogram of body weight caused some reduction in trichostrongylid (mostly *Haemonchus contortus* and Trichostrongylus spp.) egg counts. However, 50 to 75 mg./kg. of cadmium iodide and 100 mg./kg. of cadmium chloride did not.

Cadmium iodide and cadmium oxide at the rate of 100 mg./kg. eliminated all *Strongyloides papillosus* eggs, and cadmium chloride at the same rate reduced the egg count from 10,500 eggs per gram (e.p.g.) to 150 e.p.g. Cadmium iodide at the rate of 75 mg./kg. reduced the egg count from 30,300 e.p.g. to zero, while at the rate of 50 mg./kg. the count was reduced from 15,600 to 900 e.p.g. Eggs were still absent three to four months after treatment in 2 lambs given 100 mg./kg., but 300 e.p.g. were observed one month after treatment in a third lamb.

No serious signs of intoxication were observed in the treated lambs. There was, however, slight diarrhea in 4 of the animals following treatment with cadmium iodide, and marked diarrhea in a fifth lamb given 100 mg./kg. of this compound. The 3 lambs treated with 100 mg./kg. of cadmium iodide lost an average of 6.7 lb. after treatment, and the 1 treated with 100 mg./kg. cadmium oxide lost 5.0 lb., but those treated with 50 and 75 mg./kg. cadmium iodide did not lose weight significantly.

DISCUSSION

Despite the relatively small number of sheep used in this study, it is clear that all three cadmium compounds studied have a marked effect on *Strongyloides papillosus*. Cadmium iodide and cadmium oxide may also have some relatively slight effect on

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TABLE 1—The Effect of Cadmium Compounds on Gastrointestinal Nematodes of Sheep

	Pretreatment				3–5 days post-treatment			
		No. eggs per gram feces				No. eggs per gram feces		
Sheep No.	Weight (lb.)	Trichostrongylid	Strongyloides	Treatment (*per os* in capsule)	Weight (lb.)	Trichostrongylid	Strongyloides	Diarrhea
6826	59	18,600	15,600	50 mg./kg. cadmium iodide	58	23,100	900	Slight
7072	59	21,300	30,300	75 mg./kg. cadmium iodide	58	17,100	0*	Slight
7039	58	8,400	11,100	100 mg./kg. cadmium iodide	51	300	0†	Marked
6826	58	23,100	900	100 mg./kg. cadmium iodide	53	2,400	0†	Slight
7072	58	17,100	0	100 mg./kg. cadmium iodide	50	6,900	0*	Slight
19	36	12,300	3,600	100 mg./kg. cadmium oxide	31	3,300	0	None
0	....	1,500	10,500	100 mg./kg. cadmium chloride	....	1,350	150	None
6861	56	15,000	46,800	None	56	4,200	24,000	None
12	52	3,600	1,200	None	56	3,900	1,200	None
7072	60	16,500	24,900	None	59	21,300	30,300	None

* 300 Strongyloides e.p.g. present 1 month later. † Strongyloides eggs still absent three to four months later.

the gastrointestinal trichostrongylids, but it is not great enough to be important. It is likely, however, that the study of other cadmium compounds may reveal some which are effective against these nematodes. This is suggested by the fact that cadmium compounds have now been discovered to have activity not only against Strongyloides but also against such unrelated nematodes as horse strongyle larvae, Ascaris, Parascaris, and Trichinella.

The circumstances of the present study precluded necropsy of the sheep and counting of the nematodes remaining after treatment. This will have to be done to prove incontrovertibly that the worms were actually killed and not merely sterilized. Further studies of this type are planned for the future. However, the fact that no eggs were found as long as three to four months after treatment indicates that Strongyloides had been eliminated.

SUMMARY

Strongyloides papillosus eggs were eliminated from the feces of lambs treated *per os* with capsules of cadmium iodide, 75 to 100 mg. per kilogram of body weight, and cadmium oxide, 100 mg./kg. Eggs were still absent three to four months later in 2 lambs treated with 100 mg./kg. of cadmium iodide, but a few were present one month later in a third animal. Cadmium chloride given at the rate of 100 mg./kg. eliminated the majority of *S. papillosus* eggs.

Cadmium iodide and cadmium oxide also appeared to have some slight effect against gastrointestinal trichostrongylids in the treated lambs.

Diarrhea and loss of weight were observed in some of the treated animals, but these were not serious.

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Carbon Tetrachloride Poisoning of Ewes on a Low Selenium Ration

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POISONING by the halogenated hydrocarbons, characterized by hepatic centrolobular hemorrhagic necrosis, has long been recognized in both human and veterinary medicine. The use of carbon tetrachloride as an anthelmintic has frequently resulted in poisoning of the animals treated.

Species of animals vary considerably in their susceptibility to poisoning by carbon tetrachloride.[2] Susceptibility is particularly high in swine and is frequent in cattle; occasionally sheep are susceptible. Although the irregular occurrence of susceptibility in sheep is recognized, the reason for this phenomenon has not been defined.

Recent studies on *(a)* the role of selenium in dietary hepatic necrosis in the mouse, rat, rabbit, and pig [6] and white muscle disease in sheep; [5] *(b)* the interrelationship of selenium and vitamin E; [7,8] and *(c)* the protection given by vitamin E for carbon tetrachloride poisoning in the rat [3] indicated that selenium might afford protection against carbon tetrachloride poisoning.

MATERIALS AND METHODS

Two lots of 11 ewes each were fed a ration of Ladino clover hay having a selenium content of about 0.05 p.p.m. at the rate of 4.0 lb. per ewe per day, plus 0.25 lb. oats per ewe per day. In addition, sheep in lot 2 were fed 0.1 p.p.m. of selenium, calculated on a dry weight basis for the entire ration, which was given as sodium selenite mixed with the oats. These crossbred ewes averaged about 150 lb. in weight.

After 150 days on the ration, during which time they gave birth to and nursed lambs, ewes in both groups were each given 1 ml. of carbon tetrachloride orally in a soft gelatin capsule, the usual treatment for liver flukes. At this time the lambs, having been used in another experiment, were no longer with them.

RESULTS

Thirty hours after treatment, several ewes in lot 1 refused feed and 1 (No. 8) died at about forty hours after treatment. At the feeding time 42 hours post-treatment, all ewes in lot 1 refused feed and were somewhat depressed, but they improved gradually during the next 60 hours when the remaining ewes consumed a normal ration. The ewes in lot 2 remained apparently normal throughout the entire period.

At necropsy, ewe 8 had approximately one half liter of sanguineous fluid in the peritoneal cavity. The liver presented a typical picture of carbon tetrachloride poisoning with marked parenchymatous swelling, centrolobular hemorrhage, and friability. The gallbladder was turgid, the wall edematous and hemorrhagic. Microscopic examination of liver slides revealed typical centrolobular hemorrhagic necrosis (fig. 1, 2).

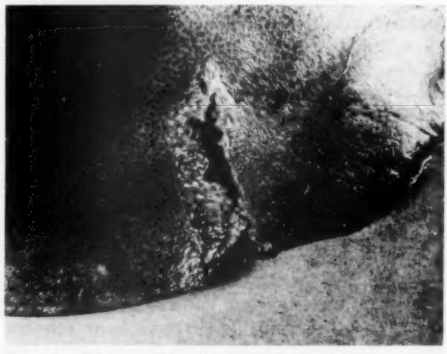

Fig. 1—Sheep liver showing centrolobular hemorrhagic necrosis.

DISCUSSION

It is of interest that the "massive liver necrosis in sheep" described as "closely identical" to nutritional hepatic necrosis of rats occurs on legume pastures.[1] Since white muscle disease of lambs occurs when ewes have been kept on legume pastures,[4] and has been prevented when a minute amount of selenium has been added to the ewes' ration,[5] the question naturally arises

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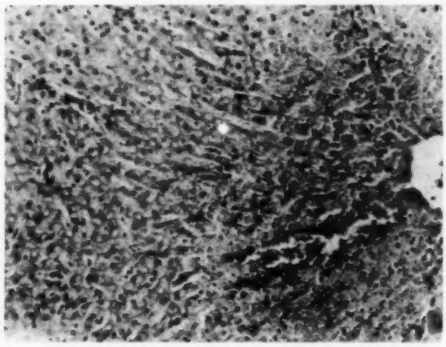

Fig. 2—Section from the centrolobular necrosis of the liver shown in figure 1. H & E stain; x 67.

as to whether massive liver necrosis in sheep is similar to nutritional hepatic necrosis of rats and other animals [6] resulting from selenium deficiency.

The results of this experiment indicate that a deficiency of selenium in ewes may cause them to be more susceptible to carbon tetrachloride. It also suggests the desirability of further investigations into the role of this element in hepatic necrosis in sheep and other species, including man.

SUMMARY

Eleven ewes on a low selenium intake developed symptoms of poisoning, and 1 died when 1 ml. of carbon tetrachloride was administered orally. The characteristic lesions of carbon tetrachloride poisoning, including hepatic centrolobular hemorrhagic necrosis, occurred in the ewe that died.

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Experimental Edema Disease (Hemolytic *Escherichia coli* Toxemia) in Pigs and in Mice

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IN THE 20 years since Shanks [13] described edema disease in northern Ireland, it has been recognized as a serious swine problem in many countries of Europe and in North America. The cause was obscure, however, until 1950 when Timoney [16] proved it to be an intestinal toxemia by reproducing the typical syndrome through the intravenous administration of supernatant fluids of the intestinal contents of pigs which had died of the disease.

In the past four years, many reports [1, 3–6, 8–10, 12, 14, 17, 18] have supported the role of toxin(s) elaborated by hemolytic *Escherichia coli* serotypes as the prime etiological factor(s) in edema disease. Sojka *et al.*[14] in England, Timoney [16] in Ireland, and the writer have found that hemolytic *Esch. coli* are consistently present in great numbers in the intestinal tracts of affected swine. Typical signs and lesions have been produced by the above authors with toxic products of hemolytic *Esch. coli* introduced parenterally. Timoney [17, 18] has prevented the disease from developing in experimental pigs by mixing an antihemolytic *Esch. coli* serum with supernatant fluids of potent edema disease-producing bowel content prior to intravenous injection.

Gregory [5] in 1957 reported that "although various serologic types (of hemolytic *Esch. coli*) had been isolated in different outbreaks, it appears that in any given outbreak the isolates from different specimens are serologically identical." In the same year, Sojka *et al.*[14] reported that the majority of their hemolytic *Esch. coli* isolations from affected pigs were represented by two serotypes. Sojka and Lloyd [15] also stated that although their group had identified nine different serotypes among 241 strains representing 229 herd cases, only three serotypes occurred commonly. In 1958, Ewing *et al.*[2] reported on the serological properties of 38 hemolytic *Esch. coli* cultures supplied by the writer and Dr. John Timoney, Veterinary Research Laboratory, Department of Agriculture, Dublin, Ireland. Although eight different serotypes were identified among the cultures, the majority were represented by two heretofore undescribed types which were assigned the group designations of 0139:K82(B):H1 and 0138:K81(B):H14 or its nonmotile counterpart. In a recent personal communication, Miura [11] of Japan informed the writer that an outbreak of edema disease in that country yielded hemolytic *Esch. coli* of O group 139.

This paper describes the experiences of the writer in the experimental reproduction of edema disease in pigs and in white mice, and in the use of neutralizing antiserums in the prevention of expressions of experimental edema disease.

MATERIALS AND METHODS

Antigens.—Hemolytic *Esch. coli* antigens were prepared by modifications of the method of Erskine *et al.*[1] with three strains (No. 15, 16, 30) of 0139:K82(B):H1 and two strains (No. 23, 31) of 0138:K81(B):H14. Antigens of strains No. 15, 16, and 23 were prepared by seeding young broth cultures to a medium of Bacto Heart Infusion broth* plus 2 per cent agar-agar and 1 per cent Bacto Tryptose.* The medium was distributed in large bottles resembling Roux flasks, and incubation was carried on at 37 C. for varying periods. Sterile saline solution (150 ml.) was used to wash the growth from the surface of 1 liter of medium. The bacterial suspensions were either alternately frozen and thawed eight to 12 times prior to centrifugation at high speed so as to obtain clear supernatant fluids, or the suspensions were centrifuged without resorting to the freezing and thawing process.

Extracts of strains 30 and 31 were prepared by growing the organisms on a medium of Bacto Dextrose Proteose No. 3 Agar * plus 0.5 per cent Bacto Tryptose. After 72 hours' incubation at 37 C., the growth was removed, suspended in saline solution, and the suspensions were frozen and thawed 12 times prior to centrifugation.

Potent bowel content extract antigens were prepared during March, 1957, from the dead pigs of two herds from which hemolytic *Esch. coli* 0139:K82(B):H1 (strains 15, 16) had been isolated. The small intestines were removed from the dead pigs and the contents collected by stripping

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* The mediums are products of Difco Laboratories, Detroit, Mich.

with the fingers. Additional material was recovered by placing 50 to 75 ml. of saline solution in the intestinal lumens and again stripping. The suspensions were then frozen for at least 24 hours. After thawing, they were centrifuged at high speed and the supernatant fluids removed, bottled, and kept frozen except for short periods when portions of extract were removed. Bowel extracts were also prepared from the intestinal contents of two herds from which hemolytic *Esch. coli* 0138:K81(B): H14 had been isolated. These extracts, however, were entirely impotent when injected into test pigs.

Potent bowel extracts A, B, and C were derived from the dead pigs of one herd and extract R from the pigs of another herd.

Antiserums.—Anti-hemolytic *Esch. coli* serum No. 15 was prepared from a cow by giving three intravenous injections of a formalized suspension of culture No. 15, 0139:K82(B):H1, at weekly intervals. This was followed by two intravenous injections of live organisms at weekly intervals. Bleeding and preparation of the serum was accomplished ten days following the last injection. Antiserums 16a and 23a were prepared from 2 steers** that had been given injections of extracts of culture No. 16, hemolytic *Esch. coli* 0139:K82(B):H1, and culture No. 23, hemolytic *Esch. coli* 0138:K81(B):H14, respectively, over a period of four months. Antiserums 16b and 23b were prepared from the same animals given the same extracts over a period of approximately 16 months.

Anti-hemolytic *Esch. coli* serums F^1 and F^2 and E^1, E^2, and E^3 were prepared by injecting extracts of culture No. 15 into 2 pigs over periods of up to five months. (The alphabetical designations refer to the pigs, while the numerical suffixes indicate the time of bleeding, *i.e.*, first, second, and third.) Anti-hemolytic *Esch. coli* serums KWP and KBP were prepared by injecting extracts of culture No. 31, 0138:K81(B):H14 into 2 pigs; 10 subcutaneous injections over a period of five months to pig KBP and 11 over a six-month period to pig KWP.

Bowel extract antiserums A1, B1, and C1 were prepared by injecting, into 3 pigs, bowel extracts derived from the pigs of the herd from which culture No. 15 was isolated. Each pig was given 12 or 13 subcutaneous injections over a three-month period.

PROCEDURE AND RESULTS

Experiments with Hemolytic Escherichia coli Extracts in Pigs.—Thirty pigs, approximately 40 lb. in weight, were injected intravenously with hemolytic *Esch. coli* extracts. Several of the pigs were also injected with anti-hemolytic *Esch. coli* serum No. 15 (table 1). Although Erskine *et al.*[1] and Timoney[19] were able to reproduce edema disease in typical form by the intravenous injection of similar materials, the experiments herein reported were disappointing, in that signs and lesions were not typical of the disease as seen under field conditions. Disturbances produced were more likely due to endotoxins unrelated to the edema disease syndrome, than to specific toxin(s) responsible for edema disease.

Immediately following administration of antigens, the pigs generally exhibited signs of severe depression; 2 died during injection and 5 died between three and 22 hours postinjection. The stomach walls of 4 of the latter 5 pigs were slightly edematous. The most prominent lesions were severe intestinal hyperemia, edema of the gallbladder, and edema and hyperemia of the colon. While the results were not clear, several pigs given the antiserum developed milder clinical signs than did similar pigs not given serum.

Effect of Bowel Extracts and Antiserums in Pigs.—Between June 15, 1957, and Dec. 22, 1958, 51 pigs of various weights were injected intravenously with lethal quantities of potent bowel content extracts. In addition, 35 of the 51 pigs were given anti-hemolytic *Esch. coli* serums and 4 were given bowel extract antiserums. All of the pigs inoculated with bowel extract alone developed severe clinical signs characteristic of field cases of edema disease, and 11 died. Necropsies revealed typical lesions such as edema of the stomach wall, subserosal edema between the coils of the colon, and excessive amounts of peritoneal fluid containing fibrin threads. The edematous areas of the stomach walls were usually at least ½-inch thick. One pig recovered following an intramuscular injection of prednisone.

The 4 pigs that were injected with bowel extract antiserums, mixed with potent bowel extract, survived the inoculations without displaying any signs of edema disease. Anti-hemolytic *Esch. coli* serums 16a, 23a, and E^1 appeared to be impotent; all of the 10 pigs which were given one of these antiserums died of typical edema disease. The other serums were generally effective in neutralizing the edema disease-producing factors when the serums and bowel extract were mixed prior to intravenous injection. Of the 16 pigs given one of these anti-hemolytic *Esch. coli* serums intra-

** Steer serums were prepared by Dr. J. D. Ray, laboratory director, Affiliated Laboratories Corp., White Hall, Ill.

venously at the time of bowel extract administration, 14 were unaffected. It is highly probable that the dosage of serum given the 2 pigs that died was inadequate.

When the antiserums were administered other than intravenously, unless given in increased amounts for a considerable time before bowel extract administration, effective neutralization of edema disease toxin(s) was not evident. In the case of 2 pigs averaging about 10 lb. in weight, 5 ml. of serum E^2 satisfactorily neutralized 5 ml. of bowel extract "B" when the products were mixed just prior to intravenous injection. In 3 other pigs of the same weight, 10 ml. of serum E^2 given intraperitoneally ten minutes before the intravenous administration of 5 ml. of the same bowel extract did not

TABLE 1—Effects of Hemolytic Esch. coli Extracts Alone or with Anti-Hemolytic Esch. coli 0139:K82(B):H1 Serum on 30 Pigs Averaging 40 Lb.

Pig	Antigen	Antiserum	Results and notations
1, 2	5 ml. 15b	None	Anorexia, some ataxia for 24 hr.; snouts, feet, ears deep purple; recovered at 48 hr.; another 5 ml. at 48 hr.; little effect.
3	4 ml. 15b	None	Washed deposit of 4 ml. resuspended; marked reaction in seconds; hyperemia, spasms; gaunt, dull for 48 hr.
4	4 ml. 23b	None	Ataxia in 1 hr.; walk backward; in 24 hr. gaunt, dull, but eating; recovered.
5	10 ml. 15b	None	Staggering in 3 hr.; soon died; stomach edema; hyperemia of stomach and intest.; marked edema, gallbladder.
6	10 ml. 15b	None	Ataxia at 8 hr.; died in 22 hr.; no edema, severe stomach, intest. hyperemia.
7	10 ml. 15b	10 ml. No. 15	Serum i.p. 16 hr. pre-antigen; slight reaction.
8	10 ml. 15b	30 ml. No. 15	Serum i.p. 24 hr. pre-antigen; slight reaction.
9	10 ml. 15b	20 ml. No. 15	Serum i.p. 17 hr. pre-antigen; dull, ataxia in 3 hr.; convulsions in 24 hr.; unable to stand at 48 hr.; became blind.
10	10 ml. 15b	10 ml. No. 15	Serum i.p. pre-antigen; slight reaction.
11	10 ml. 15b	10 ml. No. 15	Serum-antigen mix. 6 hr. in refrigerator; depressed, ataxia in 3 hr.; recovered 24 hr.
12, 13	10 ml. 23b	10 ml. No. 15	Serum-antigen mix.; 1 hr. 37 C.; both staggering in 3 hr.; 1 in convulsions in 16 hr.; both ate in 24 hr.; recovered
14, 15	10 ml. 16	10 ml. No. 15	Serum-antigen mix.; 1 died during injection; 1 ataxia in 3 hr.; dead in 5 hr.; edema of stomach and gallbladder, hyperemia stomach and intest.
16, 17	10 ml. 23b	None	One died during inject.; other, slight reaction for 2 hr.; dead at 18 hr.; edema stomach, gallbladder.
18	10 ml. 15a	None	Serum-antigen mix. 18 hr. preinjection; both depressed several hr.; normal at 24 hr.
19	10 ml. 15a	10 ml. No. 15	
20	10 ml. 15c	None	Depressed several hr.; recovered in 24 hr.
21	10 ml. 15c	None	Antigen boiled 15 min.; results same as pig 20.
22	5 ml. 15d	None	Very depressed postinject.; dead 22 hr.; slight edema of stomach; marked edema and hyperemia of colon and cecum.
23	5 ml. 15d	None	Very depressed for 24 hr.; partial paralysis jaw muscles for 2 wk.; gradual recovery.
24, 25	5 ml. 15d	None	Antigen boiled 10–15 min.; both depressed several hr.; recovered in 24 hours.
26, 27	5 ml. 15d	10 ml. No. 15	Serum-antigen mix.; 1 hr. at 37 C. pre-inject.; both depressed several hr.; recovered in 24 hr.
28	5 ml. 15d	None	Given 10 ml. antigen 23b i.p. 18 and 17 days before inject. with 15d; mild depression after latter; recovered in 24 hr.
29, 30	10 ml. 15d	10 ml. No. 15	Antigen-serum mix.; severe depression 48 hr.; gradual recovery.

Antigens—hemolytic *Esch. coli*: 15a, supernatant fluid from 24-hour agar growth of strain No. 15 suspended in saline solution; 15b, supernatant fluid from 48-hour agar growth of strain No. 15 suspended in saline solution and subjected to alternate freezing and thawing; 15c, same as 15b but not subjected to freezing and thawing; 15d, supernatant fluid of a seven-day agar growth of strain 15 suspended in saline solution but not subjected to alternate freezing and thawing; 16, supernatant fluid from 48-hour growth of strain 16, suspended in saline solution and subjected to alternate freezing and thawing; 23b, supernatant fluid from 48-hour growth of strain 23, suspended in saline solution and subjected to alternate freezing and thawing.

Antiserum: 15, prepared by injecting cow with hemolytic *Esch. coli* strain 15.

afford protection and all 3 pigs died. Of 3 pigs given 30- to 50-ml. quantities of antiserum 23b subcutaneously 24 to 72 hours before the intravenous injection of lethal quantities of bowel extract, 1 pig survived without reaction, another showed a slight reaction on the third day only, and the third seemed well until found dead on the third day.

Two additional pigs died during administration of serum-antigen mixtures and another developed such severe shock when only 2 ml. of the mixture had been injected that the experiment was terminated.

There was considerable variation in the time required for edema disease to develop in the pigs following injection of bowel extract. The size of both the dose and the pig had some influence, but this was not consistent. Many were injected late in the afternoon and, since they were not again observed until some 14 hours later, it was not known when clinical illness started. The earliest was in 50-lb. pig No. 5 which became severely ill within four hours after inoculation with 20 ml. of antigen "C" and 10 ml. of antiserum No. 15. Others given smaller doses of bowel extract remained well for at least 48 hours.

Bowel extract antigen "B" was exceptional in that it was essentially nontoxic except for its edema disease-producing properties. Most of the pigs injected with this material either displayed no signs or only mild signs of intoxication immediately following injection. Antigen "A" was exceptionally toxic. Of the 3 pigs given this extract, 1 died during injection, 1 developed severe shock when only 1 ml. had been injected, and the third was ill for several hours after injection of 8 ml. Pigs given 5 to 10 ml. of antigen "R" developed moderate shock; 1 given 20 ml. plus 25 ml. of serum, KBP developed marked shock and died several minutes later.

Points of importance in this study involve the serologic type of hemolytic *Esch. coli* isolated from the pigs from which the bowel extracts were prepared as well as the serologic types of organisms used in the preparation of the antiserums. Only hemolytic *Esch. coli* 0139:K82(B):H1 was isolated from the pigs from which the potent bowel extracts were prepared. Thus, the edema disease-producing properties of the bowel extracts were presumedly derived from organisms of the same serologic type. Of the antiserums showing evidence of edema disease-neutralizing antibodies, E^2, E^3, F^1, and 16b were anti-hemolytic *Esch. coli* 0139:K82(B):H1 serums, and 23b and KWP were anti-hemolytic *Esch. coli* 0138:K81(B):H14 serums. It would thus appear that the edema disease toxin(s) produced by these two types of organisms are antigenically similar or identical.

Mice Experiments.—Since experimentation with pigs is expensive, it was deemed advisable to investigate the suitability of white mice for edema disease investigations. Lamont [7] had reported that they had become paralyzed following intravenous injections of bowel supernatant fluids, but he was uncertain of the significance of these reactions.

In a preliminary experiment, 6 mice were used. Mouse 1, given 0.25 ml. of extract "B" intraperitoneally, moved slowly and refused food on the third day and was found dead on the fourth day. Necropsy revealed a subcutaneous edema of the abdominal wall about ⅛ inch thick.

Mouse 2, injected intravenously with 0.1 ml. of extract "B," in about 18 hours developed alternate periods of running "fits" which ended in severe tonic spasms and the mouse died with the body in rigid tonic spasm. There was no evidence of edema on necropsy. Mouse 3, given 0.2 ml. of bowel extract intraperitoneally, appeared nervous on the third day, moved slowly, and refused food. When prodded, it emitted peculiar squeaks and did not run as would normal mice. On the fourth day its abdomen seemed swollen and on the fifth day it was killed. Necropsy revealed subcutaneous edema over the hips and lateral and ventral belly walls approximately ¼ inch thick.

Mice 4 and 5, given 0.2 ml. of bowel extract intravenously, died during the following night. Subcutaneous edema was not apparent. Mouse 6, given 0.25 ml. of the extract intraperitoneally, was found dead on the third day with no evidence of subcutaneous edema.

Because of these promising results, further tests were made with 80 mice in which bowel extract "A" or "B" was injected intraperitoneally or intravenously. Of the 80 mice, 21 were given bowel extract alone and the remainder were given bowel extract plus an antiserum. Unless death occurred within 24 hours, the mice

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given bowel extract alone or bowel extract plus impotent antiserum developed typical nervous signs or edema, or both, within 24 to 48 hours. Excitability, depression, and dyspnea were generally noticed first, and during the early stages many would exhibit trembling, tonic spasms, and convulsions if disturbed. The dyspnea was undoubtedly caused by the accumulation of fluid (up to 0.7 ml.) in the thoracic cavity. If the mice survived for several days after injection of the toxic material, some tended to recover from the nervous signs and dyspnea and then develop subcutaneous edema.

Except for KWP, the anti-hemolytic *Esch. coli* serums were completely effective in neutralizing the toxicity of the bowel extracts when the two products were mixed prior to intraperitoneal inoculation. The ineffectiveness of serum KWP was surprising as it had been prepared in the same manner, except for one additional inoculation of the pig, as serum KBP which was effective in both mice and pigs. Anti-hemolytic *Esch. coli* serums 16a and 23a were unexpectedly effective in protecting mice against the effects of bowel extracts. These serums were unimpressive in protecting pigs. Three additional unrelated serums (anti-hog cholera serum, anti-swine erysipelas serum, and *Clostridium perfringens* type B, C and D antitoxin) did not protect mice from the effects of bowel extract inoculations.

Tests to determine the effectiveness of potent antiserums given intraperitoneally when the bowel extracts were injected intravenously were made with 6 mice. The 4 mice given the serum 24 hours prior to the bowel extracts were completely protected. Of the 2 given the serum half an hour before the bowel extract, 1 survived without reaction, and the other developed typical nervous signs and died.

In additional experiments, mice were given five weekly, intraperitoneal injections of hemolytic *Esch. coli* extracts in attempts to immunize them against the effects of bowel extracts. Four were given 0.1 ml. of an extract of strain No. 31 (0138:K81(B):H14) and 10 were given 0.1 ml. of an extract of strain No. 30 (0139:K82(B):H1). One week following the last injection these mice and 5 control mice were each given 0.2 ml. of bowel extract "B" intravenously. Two of the mice given bacterial extract No. 31 and 5 mice given extract No. 30 survived the experiment, indicating some protection had been afforded by the immunization procedure. One of the control mice was unaffected; however, since no other mouse had remained unaffected when injected either with bowel extract alone or in combination with impotent antiserums, this mouse may have escaped inoculation.

In a similar experiment over a period of six weeks, 3 mice were given four intraperitoneal injections of 0.1 ml. of bacterial extract No. 31 and 3 were given 0.1 ml. of bacterial extract No. 30. Ten days after the last injection, each mouse was challenged with 0.3 ml. of bowel extract "B" intraperitoneally. Five survived, 4 without evidence of illness, and 1 died. It had been injected with bacterial extract No. 31, had developed nervous signs in 24 hours, and died around 30 hours after challenge. One of the mice injected with extract No. 30 developed moderate nervous signs in 48 hours but recovered in another 24 hours.

To determine the effect of prednisone on mice injected with potent bowel extract, 1.0 mg. of the drug was given intraperitoneally immediately following an intraperitoneal injection of 0.3 ml. of bowel extract to 1 mouse and 24 hours following the extract injection to another. Both mice developed dyspnea and died. Four mice which had survived previous experimentation were also injected with 1.0 mg. of prednisone intraperitoneally and survived without incident.

DISCUSSION

There is little doubt that edema disease is a specific *Esch. coli* toxemia. The remaining essential of proof would seem to be the isolation, identification, and description of the inciting toxin(s).

Although British and Irish investigators have confirmed Timoney's[16] observation of the presence of edema disease-producing factors in the intestinal contents of pigs that have died of edema disease, this report is apparently the first originating in North America to corroborate these findings. Several North American workers have reported verbally, by correspondence, or by publication that they have been unable to reproduce edema disease with bowel content extracts. Schofield and Davis[12] were unable to reproduce the disease with filtrates prepared from pigs which died in 11 outbreaks. The impotency of some bowel

extracts may be due to the relatively minor concentration of hemolytic *Esch. coli* in the small intestine compared with that in the colon. Since the colon and cecum are generally much richer in hemolytic *Esch. coli* in diseased pigs, and since the contents of the small intestine are usually used in preparing bowel extracts, such extracts may be relatively poor in edema disease toxin(s). Timoney's reports [17,18] of the efficacy of bowel extract and hemolytic *Esch. coli* antiserums in neutralizing the edema disease toxic factor(s) have been amply confirmed in this paper. Three bowel extract and 5 hemolytic *Esch. coli* antiserums were effective in protecting pigs against the effects of edema disease-producing bowel extracts. As the anti-hemolytic *Esch. coli* serums were prepared against two distinct organism types, it seems likely that the toxic factors produced by different serotypes are antigenically similar or identical.

A syndrome in mice involving the development of nervous signs, dyspnea, and sometimes subcutaneous edema was readily produced through the administration of potent bowel extracts (Fig. 1, 2). Because of the cost factor, this finding may be of importance in future edema disease research.

Although certain serotypes of hemolytic *Esch. coli* are clearly the source of the offending toxins in edema disease, there undoubtedly are extraneous factors that determine whether these pathogenic organisms can become established in the intestinal tracts of healthy pigs. Lloyd[10] was unable to establish specific serotypes in the intestines of healthy pigs by feeding live cultures. Gregory[6] reported that a group of pigs treated orally with broth cultures of hemolytic *Esch. coli* 0139:K82(B):H1, developed edema disease seven days later, but the organisms isolated from 2 dead pigs

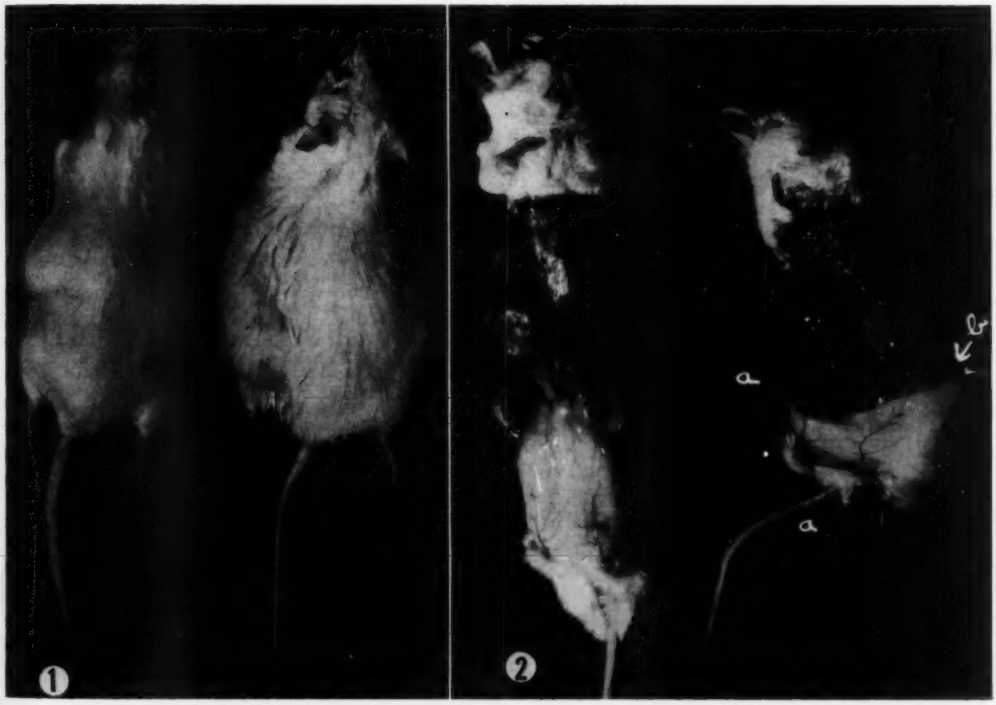

Fig. 1—Normal mouse (left) compared with edematous mouse injected with bowel extract.

Fig. 2—Same mice pictured in figure 1, with skins reflected posteriorly. Note dry-appearing surface of control mouse compared with mouse injected with bowel extract. (a) Dark area to the left of and below injected mouse consists of watery edema fluid; (b) a forceps is grasping and pulling outward an accumulation of edematous subcutaneous tissue.

were of a different serologic type. It would thus appear that conditions for the extensive growth of toxin-producing strains are usually not present in the normal healthy pig. For extensive growth of the organisms to occur, apparently certain changes must take place in the intestines. In addition, it is apparent that in some pigs the intestinal mucosa readily permits the passage of the edema disease toxin(s) into the systemic circulation, while in others comparatively little toxin(s) passes the mucosal barrier.

SUMMARY

1) Thirty pigs were given intravenous injections of extracts of hemolytic *Esch. coli*. Although all pigs developed reactions following injection, none developed typical edema disease. Of 5 pigs that died within three to 22 hours following injection, 4 showed evidence of edema of the stomach wall.

2) Of 51 pigs injected intravenously with potent bowel content extracts, derived from pigs that had died of edema disease, 35 were also injected with anti-hemolytic *Esch. coli* serums and 4 with bowel extract antiserums. Injection of bowel extract, alone, resulted in typical edema disease.

Antiserums were generally effective in neutralizing the edema disease-producing properties of the bowel extracts when the serums and extracts were mixed prior to injection. When the bowel extracts were given intravenously and the serums by other routes, it was necessary to increase the quantity of antiserums and to give them for considerable periods before bowel-extract administration in order to achieve protection.

3) In experiments with 113 mice, using potent bowel extracts intravenously or intraperitoneally, signs and lesions comparable with those of edema disease in pigs were produced. Anti-hemolytic *Esch. coli* serums were generally effective in neutralizing bowel extracts whereas anti-hog cholera serum, anti-swine erysipelas serum, and *Clostridium perfringens* type B, C, and D antitoxins were ineffective. Mice were also afforded considerable protection against the effects of bowel extracts by immunizing them with hemolytic *Esch. coli*. extracts.

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Experimental Leptospirosis. VII. Re-Exposure of Pregnant Sows with *Leptospira pomona*

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LEPTOSPIROSIS, due to *Leptospira pomona* infection, has been demonstrated by experimental and clinical studies to be an important cause of porcine abortion and the birth of weak pigs.[1,2,4,5] The pregnant sow appears to be most susceptible to leptospirosis during the last half of gestation.[1] It has been inferred that swine previously infected would be refractive to subsequent exposure and able to complete a normal gestation period.[1] Since experimental proof was lacking, challenge of the immunity of pregnant sows was undertaken to determine if the maternal antibodies were capable of protecting the fetuses at the time of re-exposure of the sow to *L. pomona*.

MATERIAL AND METHODS

Five pregnant sows, a susceptible boar and a susceptible barrow served as experimental animals. The sows had been experimentally infected with *L. pomona* ten and 14 months previously. Urinary shedding had not been demonstrated for eight to ten months.[3] Sows were bred by natural service 75 to 95 days prior to re-exposure.

The modified agglutionation-lysis test [9] was employed with end point titers interpreted as the tenfold serum dilution in which at least 50 per cent agglutination or lysis occurred. Serum antibodies for *L. pomona* were demonstrated in the sows at end point titers of 10^3 and 10^4. Antibodies were not present in the serums of the boar and barrow.

The challenge strain of *L. pomona* of porcine origin had been maintained in continuous guinea pig passage in this laboratory. The inoculum consisted of heparinized hamster blood and a 10.0 per cent suspension of hamster kidney and liver tissue in 0.85 per cent sodium chloride solution. Four of the sows, the boar, and the barrow each were given 7.5 ml. of the mixture. Titration of the inoculum in guinea pigs indicated the presence of at least 10^5 leptospires per milliliter. Sow 4928 served as a control and was given 7.5 ml. of normal hamster blood and tissues.

Clinical observations were made and rectal temperatures were taken twice daily for ten days. Blood cultures were prepared using 0.1 ml. of blood inoculated into each of five tubes of Chang's medium on postexposure days 5 to 8. These were examined by dark field microscopy at weekly intervals for one month or until proved positive.

All swine were anesthetized by intravenous administration of sodium pentobarbital, exsanguinated, and postmortem examinations conducted. Fetal and maternal kidneys, livers, spleens, brains, adrenal glands, hearts, and placentas were fixed in 10 per cent buffered formalin, Zenker's, and Carnoy's solutions. The stains used were hematoxylin and eosin, Heidenhain's aniline blue, periodic acid-Schiff, Sudan IV, and Warthin-Starry silver impregnation.

Guinea pigs were inoculated with tissue suspensions of kidney, liver, spleen, brain, mammary gland, uterus, and testes from the mature pigs, as well as fetal kidney, liver, and spleen tissue suspensions. Urine and amnioallantoic fluids were also inoculated into guinea pigs. Positive serum titers (determined by a modified agglutination-lysis technique [9]) in guinea pigs 18 to 21 days after inoculation were considered indicative of the presence of leptospires in the various tissues.

RESULTS

None of the pregnant sows, control or challenged, evidenced any clinical signs of leptospirosis. The boar and the barrow had temperature rises to 104.6 F. on postinfection days 5 and 6, with concurrent constipation and anorexia.

Leptospires were isolated from the blood of the boar and the barrow by culture methods on postinfection days 5, 6, and 7. Isolations were not accomplished from the blood of the pregnant sows. By guinea pig inoculation, leptospires were demonstrated in the kidney and brain of the barrow on postinfection day 7, but not from the liver or spleen. Leptospires were present in the kidney of the boar on postinfection day 13; all other tissues, including the testes, were negative.

The control sow (4928) was killed on day 3. Maternal kidney, liver, and spleen tissues, as well as amnioallantoic fluid, fetal blood, and fetal kidney and liver

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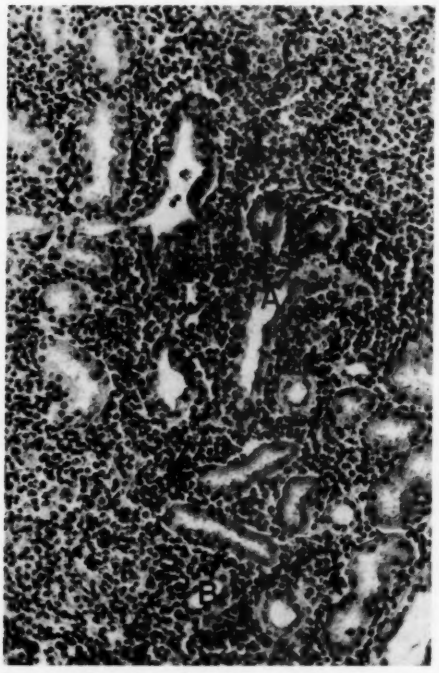

Fig. 1—Marked infiltration of lymphocytes in cortex of a kidney. (a) Atrophy and disappearance of tubules; (b) pyknosis of nuclei of tubular epithelium. x 130.

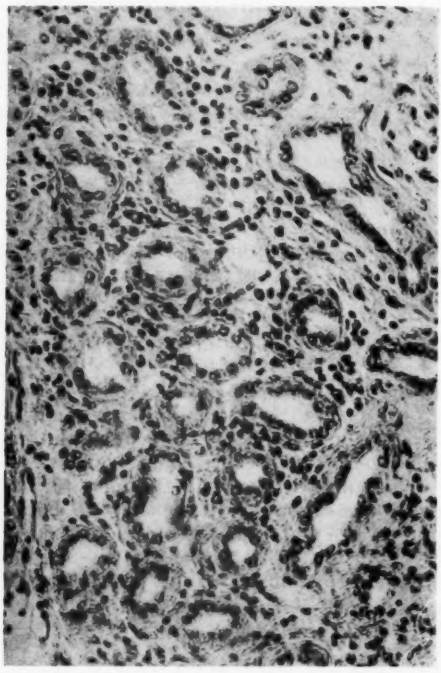

Fig. 2—Extensive increase of fibrous connective tissue and some lymphocytes between tubules of medulla of kidney. x 130.

tissues were inoculated into guinea pigs, but leptospires were not demonstrated. Seven 90-day fetuses appeared normal. The maternal serum gave agglutination-lysis reactions of 10^4. Urine antibody was demonstrated at 10^3. The fetal serums and amnioallantoic fluids were serologically negative.

Sow 4929 was killed on postchallenge day 6. Nine apparently normal fetuses, in approximately the 95th day of gestation, were present. Fetal and maternal tissues did not contain leptospires. The terminal agglutination-lysis titer of the maternal serum was 10^7 and maternal urine, 10^3. Fetal serums did not contain antibodies against *L. pomona*.

At necropsy, 11 days after challenge, 10 apparently normal, viable, 86-day fetuses were present in sow 4958. Leptospires were not isolated from the fetuses or the sow. The end point titer of the maternal serum was 10^8; the urine titer was 10^2. Leptospiral antibody reactions were not observed in the fetal serums.

Seventeen days after challenge, sows 4923 and 4969 farrowed litters of 9 and 10, respectively, which developed normally. Agglutination-lysis reactions occurred in a 10^1 dilution of serums of newborn pigs three hours after birth. Samples procured from the pigs at birth were negative. Two pigs from each litter were killed when 48 hours old, and tissues were inoculated into guinea pigs. The results were negative.

Sow 4969 was killed on postchallenge day 41 and sow 4923 on day 61. Leptospires were not isolated from the kidneys or urine.

At necropsy, the gross changes (grayish-white foci, measuring 1 to 4 mm. in diameter), were confined to the kidneys of all the mature pigs, including the control sow. On section, a few lesions extended through the cortex into the medulla. The placenta and fetal kidneys appeared to be normal.

Microscopically, many of the foci in the challenged sows' kidneys consisted of an infiltration of lymphocytes, a few plasma cells, and some macrophages (fig. 1). Some areas were characterized by active prolif-

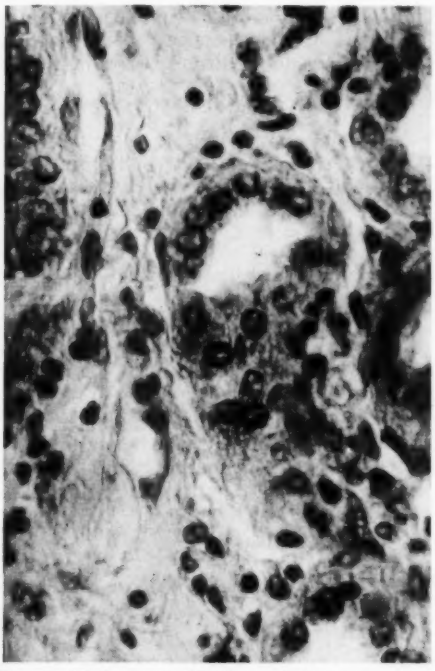

Fig. 3—Higher magnification of figure 2. x 360.

eration of fibroblasts, while others were primarily cicatricial tissues (fig. 2, 3). In these foci, a tubule was occasionally atrophied. The renal corpuscles had some thickening of Bowman's capsules and the glomerular tufts. The epithelium of the proximal convoluted tubules had an increase of fat (fig. 4). The kidney lesions of the boar and barrow were composed of small areas of predominantly lymphocytic infiltration, similar to the type described by Langham *et al.*[6]

An active inflammatory process, typified by foci of lymphocytic infiltration, was an unusual finding in the kidneys of the control sow. Some foci extended only 1 to 2 mm. below the cortical surface, while others penetrated to depths of 8 to 9 mm., reaching the medulla. Connective tissue proliferation was most marked in the medulla. No evidence of leptospires was observed in the silver-impregnated kidney sections.

Discussion

The pathogenicity of the challenge strain was adequately demonstrated by the course of the disease in susceptible swine. During the acute phase, leptospires were isolated from blood by culture methods and from kidneys, liver, and spleen by animal incubation. The demonstration of *L. pomona* in the brain tissue of the barrow indicates an invasiveness for this tissue. Encephalitis or meningitis, reported as a clinical manifestation of porcine leptospirosis,[7, 11] could result from direct invasion of the brain by leptospires.

Maternal antibodies are unable to cross the placental barrier of the sow. It had been postulated that some organisms might escape the effect of the maternal antibodies, cross the placental barrier, and survive in the antibody-free fetal environment. Since leptospires were not demonstrable in fetal materials, and abortions or fetal deaths did not occur, the maternal antibodies furnished adequate protection against challenge and uterine invasion apparently did not occur. Gestation and parturition were normal. Colostral antibodies were detected in the serums of newborn pigs three hours

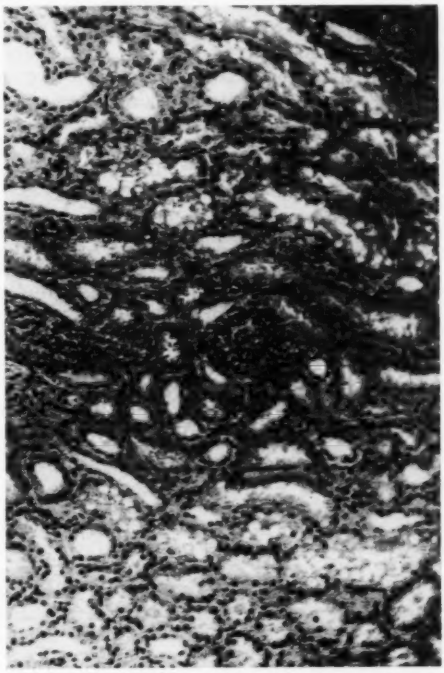

Fig. 4—Medulla of a kidney showing fat vacuoles in terminal portions of proximal convoluted tubules and some intertubular infiltration with lymphocytes. x 130.

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after birth, so protection against possible postnatal infection was afforded.

Marked increase in serum antibody levels was observed following challenge, with end point titers increasing by a factor of 10^4 in the immune sows. The response was transient; the serum titer of sow 4969 decreased from 10^8 to 10^4 by postchallenge day 41. The barrow had leptospiremia with a concurrent serum titer of 10^4 on postinfection day 7. Leptospires were isolated from this blood in the presence of homologous antibody.

The early immune response may produce an incomplete antibody, which is unable to react with all the leptospires. Leptospires can be successfully maintained through several passages in mediums containing homologous antibody.[10] This phenomenon sugests a possible mechanism for *in vivo* selection or variance. Not all leptospires are agglutinated by homologous antiserum *in vitro; i.e.*, Morse *et al.*[9] suggest that agglutination is rarely complete in the agglutination-lysis test. Neutralization of the circulating antibody permits survival of some organisms.

The histopathologic changes in the susceptible animals were comparable to renal changes previously described.[3,6] Langham *et al.*[6] described microscopic lesions of repair and active inflammatory reaction 184 days after exposure. Similar lesions were observed in the kidneys of the re-exposed sows. Active renal inflammation could have been the result of challenge; *i.e.*, the exposure of a sensitized renal tissue to the leptospiral antigen. However, this would not explain the active inflammatory processes in the kidneys of the control sow. Since active inflammatory processes persisted for ten to 14 months after initial infection, some antigenic principles of the leptospires, capable of stimulating a cellular reaction, may be retained in localized areas after renal shedding is not detectable.

SUMMARY

The immunity produced by active infection was sufficient to protect pregnant sows and their fetuses against challenge with a virulent strain of *Leptospira pomona*. Such animals should be satisfactory to retain for breeding purposes.

Active inflammatory processes appear to persist in the porcine kidney for ten to 14 months after initial infection and eight to ten months after leptospires are demonstrated in the urine.

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Studies on Five Porcine Enteroviruses

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THE INTESTINAL TRACTS of animals and human beings serve as a natural abode for viruses, just as they do for bacteria. The application of tissue culture techniques has made possible the recognition and study of many of these enteroviruses—viruses whose primary habitat is the intestinal tract. A current problem of the virologist is to differentiate these enteroviruses and to determine their disease-producing capabilities.

This report describes some of the characteristics of five antigenically different viruses which originated from the feces of swine. These viruses will be referred to in this report as ECPO (enteric cytopathogenic porcine orphan) viruses.

Previous reports on the isolation of viruses from the intestinal tracts or feces of swine by the use of tissue culture methods are rather limited. Moscovici *et al.*,[8] from Italy, reported the isolation of a virus from a newborn pig with enteritis. Beran *et al.*[1] isolated a number of viruses with the majority belonging to a single homotypic group. Previous reports from our laboratory [4,9] have described the isolation of 30 unidentified viruses from swine in eight herds, with preliminary studies indicating four different virus types; and the use of the plaque technique for the study of the porcine enteroviruses.

MATERIALS AND METHODS

Cell Cultures.—Primary monolayer cultures were prepared by the trypsinization of the designated tissue with the subsequent growth of the dispersed cells in a culture medium consisting of: 0.5 per cent lactalbumin enzymatic hydrolysate; 3.0 to 10.0 per cent bovine serum; 96.5 to 89.5 per cent Hanks' balanced salt solution (BSS); and 100 μg. of streptomycin, 200 units of penicillin, and 50 units of nystatin (Mycostatin*) per milliliter.[4]

Plaque Technique.—Monolayer cultures of porcine kidney cells in 4-oz. prescription bottles were prepared and used as previously described.[9] The agar overlay was composed of a lactalbumin enzymatic hydrolysate medium, as described above, except that it contained 1 per cent Noble agar (Difco), 3 per cent bovine serum, and 0.002 per cent neutral red.

Virus Isolations.—Viruses were isolated from porcine feces by the use of conventional tissue culture methods [7] employing porcine kidney cells.

Serological Procedures.—Antiserums for the isolated porcine viruses were prepared in rabbits according to the procedure outlined by the Committee on ECHO Viruses.[2] Plaque-isolated strains of viruses were used in the preparation of ECPO-1 and five antiserums.

Neutralization tests for determining the antigenic relationships of these viruses were conducted by the use of the plaque technique. The basic technique was as follows: Equal volumes of the virus (diluted so that in 0.1 ml. a countable number of plaques would occur) and the diluted antiserum were incubated at room temperature for one hour. Also, as a control and as a guide to determine the extent of virus neutralization, the same concentration and volume of the virus was added to an equal volume of BSS and incubated similarly as the virus-antiserum mixture. The BSS served as the diluent for the viruses and antiserums and 0.2 ml. of each of the virus-serum and the virus-BSS mixtures was inoculated individually into a tissue culture bottle. The plaque technique was then followed. These bottles were observed daily for evidence of plaques. By comparing the number of plaques in the virus control bottles (virus + BSS) with the number in the bottles inoculated with the virus-serum mixtures, the extent of virus neutralization was readily determined.

Since the plaques produced by the ECPO-4 virus were small and indistinct, the neutralization tests with this virus were conducted by the use of tissue culture tubes as follows: Equal amounts of the diluted antiserums and the virus (50 $t.c.d._{50}$ per 0.1 ml.) were mixed and incubated at room temperature for one hour. Then, 0.2 ml. of the mixture was inoculated into each of three tubes of porcine kidney tissue culture. The tubes were then incubated at 37 C. and examined for cytopathogenic changes daily for ten days.

Poliomyelitis, Coxsackie, and ECHO antiserums* were tested for their neutralizing effects on the porcine enteroviruses. Anti-poliomyelitis serums were diluted and pooled so as to represent a final dilution of 1:10 for each of the three types. The following types of Coxsackie antiserums in the dilutions as indicated in the parentheses were

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* Mycostatin is produced by E. R. Squibb and Sons, New York, N.Y.

* Supplied by National Foundation for Infantile Paralysis Inc.

used: B 1(1:25), B 2(1:10), B 3(1:10), B 4 (1:10), B 5(1:10), A 9(1:25). The ECHO antiserums were pooled according to the method described by Melnick,[7] wherein pool A represented types 2, 3, 5, and 6; pool B, 7, 8, 9, 10, and 11; pool C, 1, 12, and 13; and pool D, 4 and 14.

CPE Spectrum.—For determining the cytopathogenic effect (CPE) of the ECPO viruses on tissue culture cells of different sources, 0.1 ml. of the viruses were inoculated into each of three tissue culture tubes which were microscopically observed daily for seven days.

as "virus 5-2,"[5] was isolated from a normal mature hog which had been hyperimmunized six days previously with hog cholera virus; ECPO-5, formerly referred to as "virus 11-45,"[4] had been isolated from an 8-week-old pig with severe diarrhea.

RESULTS

Isolation of Viruses.—Viruses were readily isolated from the feces of a high percentage of normal or sick pigs. Bacterial contamination of the tissue cultures inocu-

TABLE 1—Average Diameter (mm.) and Morphological Characteristics of the ECPO Virus Plaques

ECPO* viruses	First appearance of plaques (days)	Days				Plaque pattern
		3	5	7	10	
1	1.5–2.0	4.0**	9.0	14.0	20.0	Circular, sharp boundaries.
2	2.0–3.0	1.5	3.0	4.5	6.0	Diffuse boundaries.
3	4.0–5.0	0	1.0	2.0	3.0	Indistinct; diffuse boundaries.
4	7.0–8.0	0	0	0.5	1.0	Very indistinct.
5	1.5–2.0	4.0	9.0	14.0	20.0	Circular, sharp boundaries.

* Proposed nomenclature. ** Diameter (mm.) of plaques.

Chicken Embryo Inoculations.—Tissue culture fluid virus (0.2 ml.) of each of the five ECPO viruses was inoculated into the yolk sacs of 5- to 7-day-old chicken embryos and into the allantoic sac of 10- to 12-day-old chicken embryos. After seven days, one "blind" passage was made with each virus using either allantoic fluid or a 20 per cent suspension of yolk sac depending on the original route of inoculation. Impression smears were made from the yolk sac and stained by Macchiavello's method.

Origin of the ECPO Viruses.—All five of the ECPO viruses used in this study were isolated from the feces of swine: ECPO-1, formerly referred to as "virus 6-6,"[4] had been isolated from a shoat with diarrhea, which in all probability was caused by an infection with the virus of transmissible gastroenteritis (TGE); ECPO-2, formerly referred to as "virus 11-56b,"[4] and ECPO-3 originated from clinically healthy shoats; ECPO-4, previously designated

lated with the fecal extracts was negligible, especially if the fecal extracts had been stored at −15 C. for a few days. Most of the virus isolations were made from pigs over 5 weeks old.

Differentiation of the Isolated Viruses.—Plaque Morphology.—Four distinctly different types of plaques have been produced by the viruses isolated from porcine feces. The appearance of three types of plaques at two incubation periods is illustrated (fig. 1 and 2). The fourth type of plaque was so small that it was not readily discernible in photographs. Each virus consistently produced the same type of plaque. The diameter and pattern of these four types of plaques are indicated (table 1). For convenience, these viruses are tenta-

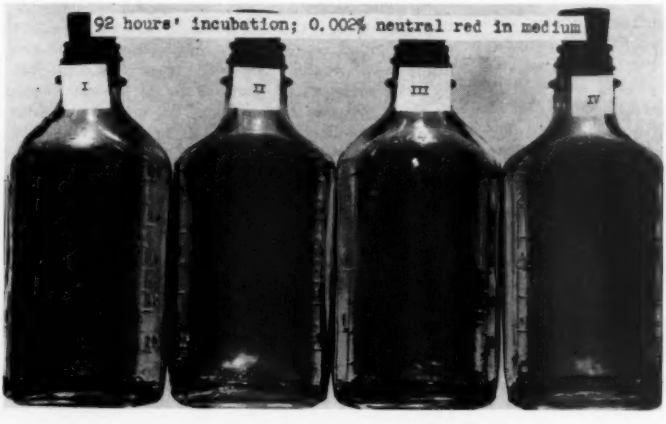

Fig. 1—Appearance of plaques after 92 hours. Bottles inoculated with virus as follows: I, ECPO-1; II, ECPO-2; III, ECPO-3; IV, control (no virus).

tively designated ECPO-1, -2, -3, or -4 viruses on the basis of plaque morphology.

Antigenic comparison.—Five antigenically distinct viruses have been detected (table 2). Those viruses which differ on the basis of plaque morphology (ECPO-1, -2, -3, -4) also differ antigenically. Another virus, designated ECPO-5, has some antigenic relationship with ECPO-1 (tables 2, 3, and 4). Also, the plaques produced by the ECPO-1 and the ECPO-5 viruses are morphologically indistinguishable (table 1).

Titration of the ECPO antiserums with their homologous virus is shown (table 5).

Neutralization Tests with Poliomyelitis, Coxsackie, and ECHO Antiserums.—The ECPO-1, -2, and -3 viruses were not neutralized by the poliomyelitis antiserums or the ECHO antiserums; ECPO-4 and -5 have not been tested. ECPO-1, -2, -3, and -5 were not neutralized by the Coxsackie antiserums; ECPO-4 has not been tested.

CPE Spectrum.—These porcine enteroviruses produced a CPE on all tissue culture cells derived from porcine tissues; their effect on other types of cells varied (table 6).

Chicken Embryo Inoculations.—None of the five ECPO viruses produced death or visible evidence of infection in the inoculated chicken embryos.

TABLE 2—Neutralization of ECPO Viruses with Homologous and Heterologous Antiserums

ECPO viruses	ECPO antiserums (each diluted 1:16)					Control (HBSS)
	1	2	3	4	5	
1	0*	11	14	15	4	19
2	40	0	43	NT	36	31
3	40	43	0	25	36	30
4**	3/3†	3/3	3/3	0/3	3/3	3/3
5	2	25	23	NT	0	30

* Number of plaques per bottle. ** Tested by tissue culture tubes; † enumerator, number of tubes showing CPE; denominator, number of tubes inoculated. NT, not tested.

DISCUSSION

This report indicates that a number of different viruses may be detected in the feces of swine by the use of tissue culture methods. So far, five antigenically different types have been isolated and it is anticipated that more will be found. These viruses should probably be considered the porcine counterparts of the human enteroviruses—poliomyelitis, Coxsackie, and ECHO viruses.

Temporarily, at least, it is proposed that the viruses isolated by tissue culture methods from the feces or intestinal tracts of swine be called ECPO (enteric cytopathogenic porcine orphan) viruses followed by a number indicating different antigenic types. Although the term ECSO (enteric cytopathogenic swine origin)[6] has been used previously in a somewhat casual fashion to refer to such viruses, the term ECPO

TABLE 3—Neutralization of ECPO-1 and -5 Viruses with ECPO-1 Antiserum

ECPO viruses	Dilutions of ECPO—1 antiserum				Control (HBSS)
	1:16	1:64	1:256	1:1,024	
1	0*	0	2	6	19
5	2	13	26	31	30

* Number of plaques per bottle.

Fig. 2—Appearance of plaques after seven days. Bottles inoculated with virus as follows: I, ECPO-1; II, ECPO-2; III, ECPO-3; IV, control (no virus).

XUM

TABLE 4—Neutralization of ECPO-1 and -5 Viruses with ECPO-5 Antiserum

ECPO viruses	Dilutions of ECPO—5 antiserum				Control (HBSS)
	1:16	1:64	1:256	1:1,024	
5	0*	0	0	0	30
1	4	6	14	16	19

* Number of plaques per bottle.

seems more suitable for the following reasons: (1) "porcine" is preferably used as an adjective and "swine" as a noun; and (2) "porcine" is more nearly analogous to "human" and "bovine" which have been used in the following accepted terms, ECHO and ECBO viruses.

The plaque technique, which helps quantitate viral agents, was used extensively in this work. Plaque morphology was helpful in differentiating these viruses. So far in our studies, viruses which produce different types of plaques are also antigenically distinct, as indicated by the ECPO-1, -2, -3, and -4 viruses. However, viruses producing indistinguishable plaques may differ antigenically; for example, ECPO-1 and -5 viruses, although they were antigenically related. Viruses that produce similar types of plaques are probably more closely related than those producing dissimilar plaques, and thus plaque morphology may be of help in classifying viruses. The ECPO-1 and -5 viruses produce plaques that are similar to those of the poliomyelitis viruses, and whether this is indicative of similar pathogenic potentiality remains to be seen. The plaque technique was found to be admirably suited for conducting neutralization tests.

These viruses have been isolated from both healthy swine and those with diarrhea. Although no disease has yet been associated with any of these viruses, their pathogenic potentialities should be considered and probably exist. Speculation on what diseases, if any, may be caused by these porcine enteroviruses leads us to make some comparisons with what is known about the human enteroviruses. The latter have been associated with such diverse illnesses as paralysis (complete to slight muscle weakness), aseptic meningitis, myocarditis, pleurodynia, summer rash, and diarrhea, although some are still "orphans," that is, not associated with any disease.[3]

TABLE 5—Titration of ECPO Antiserums with Homologous Virus

ECPO viruses	Dilutions of homologous antiserums				Control (HBSS)
	1:16	1:64	1:256	1:1,024	
1	0*	0	2	6	19
2	0	0	5	17	31
3	0	0	0	2	30
4**	0/3†	0/3	0/3	3/3	3/3
5	0	0	0	0	30

* Number of plaques per bottle. ** Tested by tissue culture tubes; †enumerator, number of tubes showing CPE; denominator, number of tubes inoculated.

TABLE 6—Cytopathogenic Effect of ECPO Viruses on Tissue Culture Cells of Different Sources

Source of cells	ECPO viruses				
	1	2	3	4	5
Porcine kidney	+	+	+	+	+
Porcine testes	+	+	+	NT	+
Porcine lung (embryo)	+	+	NT	NT	NT
Porcine intestine (embryo)	+	+	NT	NT	NT
Bovine kidney	—	—	—	NT	—
Chicken testes	—	NT	—	NT	NT
Human amnion	—	—	—	±	—
Rabbit kidney	—	—	—	±	—
HeLa cells	—	—	—	NT	—
Monkey kidney*	+	—	—	+	+

\+ indicates CPE; —, no CPE; NT, not tested. * Rhesus (*Macacus mullata*).

None of these porcine enteroviruses has yet been identified as any previously known virus. Poliomyelitis, Coxsackie, and ECHO antiserums have not neutralized any of the viruses so far tested. The ability or inability to produce a CPE on a variety of cells or to produce infection of chicken embryos may be used to rule out a number of known viruses, such as, pseudorabies, TGE of swine, hog cholera, poliomyelitis, some bovine enteroviruses, Newcastle disease, and herpes simplex.

Epidemiological studies with these porcine enteroviruses are now in progress, and it is anticipated that they will serve a useful purpose for obtaining basic information applicable to the enteroviruses in general.

SUMMARY

1) Five antigenically different viruses have been isolated from the feces of swine by the use of tissue culture methods utilizing porcine kidney cells.

2) Four of these viruses produced distinctly different types of plaques.

3) Two antigenically different, but related, viruses produced indistinguishable plaques.

4) It is proposed that these viruses, temporarily, at least, be called ECPO (enteric cytopathogenic porcine orphan) viruses,

followed by a number indicating different antigenic types.

5) No disease in swine has yet been associated with these viruses, although this aspect has not yet been pursued.

6) None of these five viruses has been identified with any previously known virus, although this possibility has not been thoroughly explored.

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Hemadsorption and Cytopathic Effect Produced by African Swine Fever Virus in Swine Bone Marrow and Buffy Coat Cultures

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Muguga, Kenya, East Africa

AFRICAN SWINE FEVER (ASF) has been compared with hog cholera (European swine fever) on a clinical and pathologic basis by several authors,[6,9,10,14,16] Although differences were observed in the clinical signs as well as in the gross and microscopic pathologic changes, these differences were not so distinctive that they would provide an unqualified differential diagnosis. The causative virus of ASF, however, is immunologically distinct from that of hog cholera, as demonstrated by cross-immunity tests.[4,6,10,16] The occasional recovery from hog cholera is followed by a solid immunity against the homologous virus but offers no protection against ASF virus. On the other hand, it is doubtful if pigs surviving the initial infection with ASF virus develop protective antibodies, since the resistance to further challenge is dependent upon a persisting viremia.[3] This immunologic distinction has been a basis for the differentiation of the two diseases, which have so many features in common.

Maurer *et al.*[9] have pointed out the need of a specific diagnostic test which will simplify the recognition of ASF, especially in areas where hog cholera is prevalent and could easily obscure the occurrence of ASF. The utilization of swine bone marrow cell cultures for the propagation of hog cholera virus [1] prompted an investigation to determine the value of these as well as buffy coat cultures for detection of ASF virus.

It is the purpose of this report to describe an *in vitro* reaction produced by ASF virus in swine bone marrow and buffy coat cultures which is believed to be specific and which has not been observed with hog cholera virus.

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MATERIALS AND METHODS

Tissue Culture Systems.—Cultures of bone marrow cells and buffy coat cells were prepared by modification of the methods described by Boynton[1] and Woodliff.[17] The femur from a freshly slaughtered pig generally provided sufficient bone marrow for all the cultures required for immediate use. After the bone marrow was harvested, an equal volume of medium 199[11] was added and the cells were released from the trabeculae by thorough mincing with scissors. The cell suspension was allowed to stand for a few minutes to permit the bone fragments and large particles to settle prior to removal of the homogeneous cellular portion with a pipette. The cell concentration was estimated by diluting the suspension in leukocyte diluting fluid and making several counts in a hemocytometer. Further dilution of the suspension was made in nutrient medium composed of 80 per cent mixture 199 and 20 per cent normal pig serum to give approximately 5×10^6 cells per milliliter. The cells were dispensed into tubes or milk dilution bottles and kept in stationary racks at 37 C. Inoculation of the cultures with ASF virus was made immediately or within three days after preparation.

Cultures of buffy coat cells were made from blood collected from the anterior vena cava directly into heparin * or 3 per cent sodium citrate. Heparin was used in a concentration of 0.2 mg. per milliliter of blood. Sodium citrate solution was used in the ratio of 1:5 parts of blood. After centrifugation and aspiration of plasma, the buffy coat was placed in washing fluid and dispersed by vigorous pipetting. When heparin was used as an anticoagulent, the washing fluid consisted of mixture 199; otherwise, saline solution free of calcium, magnesium, and phosphate was used.[8] After one washing, the buffy coat cells were resuspended in an appropriate volume of nutrient medium and the cell concentration was estimated by making cell counts as described for bone marrow cells. The cell suspension was further diluted in medium to give approximately 5×10^6 cells per milliliter and placed in tubes held in a stationary rack at 37 C. These cultures were sometimes inoculated immediately, but were generally used on the second day of incubation.

* Liquemin, produced by Roche Products Limited, Welwyn Garden City, England.

By comparison of cell counts of freshly drawn blood and suspensions of buffy coat cells, it was estimated that approximately 50 per cent of the leukocytes were recovered. Based on this recovery and an average leukocyte count for normal pigs of 15,000 to 20,000 per cubic millimeter, each milliliter of blood was expected to yield 2 ml. of cell suspension, with a concentration of 3.75×10^6 to 5.0×10^6 cells per ml. Although this may be an oversimplification, it has made possible the preparation of small numbers of culture tubes on short notice. For this purpose, 10 ml. of blood was drawn from the anterior vena cava and discharged directly into a centrifuge tube containing heparin. After centrifugation and aspiration of plasma, the buffy coat was carefully removed and, without preliminary washing, resuspended in nutrient medium to make a final volume of 20 ml. Ten tubes may be implanted with 2 ml. of cell suspension per tube and inoculated immediately with the suspect material.

Virus Isolations.—Four strains of ASF virus were used in the tissue culture systems. The locally isolated Kenya (Hinde) strain[7] was used in most of the studies. Other strains of ASF virus studied were a South African (Spencer), a northern Rhodesian, and a Portuguese (Lisbon) strain. Infectious materials consisting of spleen, whole blood, or serum were used as sources of inoculum. After one or more passages in bone marrow, buffy coat, or other tissue culture systems, the supernatant tissue culture fluids were used for additional studies.

Cover Slip Preparations.—Suspensions of bone marrow or buffy coat cells were implanted in medium in Leighton tubes containing cover slips. Control cultures were left uninoculated while the remaining cultures were inoculated immediately after implantation. Cover slips were removed from the control and infected cultures after 24 hours of incubation and at 24-hour intervals thereafter for fixation in methyl alcohol and staining with Leishman's stain.

Virus Titrations.—Dilutions of ASF virus were made in chilled medium 199, and five tubes of buffy coat cultures were inoculated with each dilution, using 0.2 ml. of inoculum per tube. All tube cultures were kept in a stationary rack at 37 C. and observed daily under low-power magnification for ten days. The end points were calculated by the method of Reed and Muench.[12]

Virus-Neutralization Studies.—Serum was obtained from survivor-carriers at different intervals after recovery from the acute phase of ASF. All serums were inactivated at 56 C. for 30 minutes and mixed with equal volumes of virus dilutions. The serum-virus dilution mixtures were incubated 37 C. and observed daily under low-power magnifibuffy coat cultures were inoculated for each dilution mixture, using 0.2 ml. of inoculum per tube. Normal serum was included in each series as a control. Observations were made daily for ten days.

RESULTS

Cultures of Bone Marrow Cells and Buffy Coat Cells.—The bone marrow cells attached rather quickly to the glass substrate, and generally went through the developmental phases described by Woodliff.[17] Several cell types were present in the cultures; however, identification and classi-

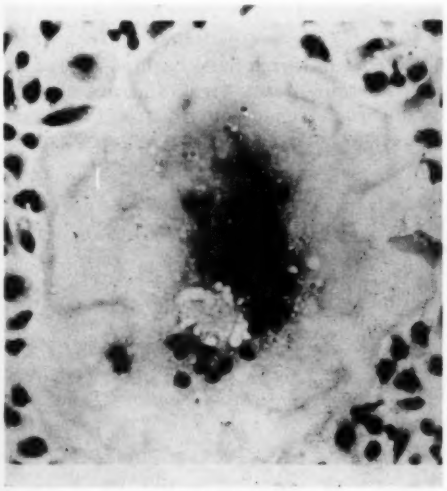

Fig. 1—Multinucleated giant cell in noninfected buffy coat culture after 72 hours of incubation. Leishman's stain; x 178.

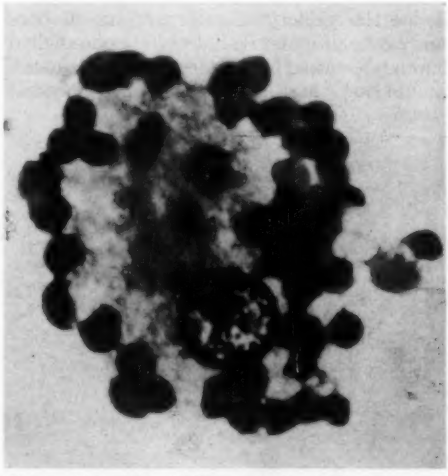

Fig. 2—Infected cell from a buffy coat culture, 72 hours postinoculation. Erythrocytes are adsorbed at the periphery and on the surface of the cell. Leishman's stain; x 900.

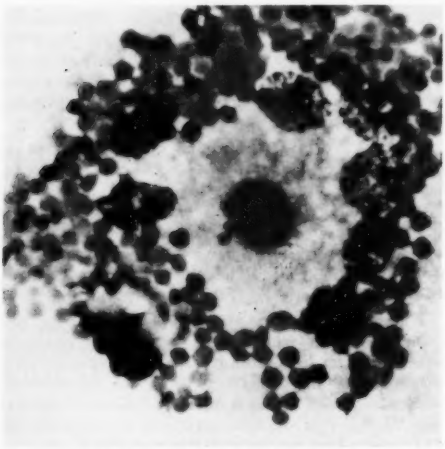

Fig. 3—Infected, multinucleated giant cell from a buffy coat culture inoculated immediately after implantation, 72 hours postinoculation. Erythrocytes are adsorbed on the surface. Leishman's stain; x 900.

fication was not attempted. Polymorphonuclear leukocytes persisted in the cultures for several days, while fibroblast-like cells appeared after approximately three days' incubation. Large granular cells corresponding to the second phase described by Woodliff [17] were observed on the second day and were phagocytic as evidenced by the large number of engulfed erythrocytes.

Cultures of buffy coat cells did not evidence the variety of cells present in bone marrow cell cultures. Polymorphonuclear leukocytes were not present after 24 hours' incubation, and only the large granular cells were found two to three days later. These cells were also phagocytic and contained many engulfed erythrocytes, the cytoplasm sometimes becoming completely filled. Giant cells (fig. 1) were formed as early as 72 hours after implantation, with some cells containing over 40 nuclei. Fibroblast-like cells appeared in scattered areas of the cultures which were kept longer than six or seven days.

Reaction to African Swine Fever Virus.—After inoculating bone marrow and buffy coat cell cultures with ASF virus, the first evidence of infection was adsorption of erythrocytes around the periphery and on the surface of large granular cells (fig. 2). Depending upon the virus concentration, this phenomenon was observed as early as 18 hours and as late as ten days postinoculation. Beginning with only a few infected cells, the number gradually increased until a majority of the cells became involved. Giant cells which became infected (fig. 3) also held adsorbed erythrocytes on the surface. The infected cells finally underwent cytolysis (fig. 4). A noninfected buffy coat culture appeared as shown (fig. 5). Fibroblast-like cells were not involved in the reaction, and continued to proliferate in cultures where all of the susceptible cells had undergone degeneration. Preliminary adaptation of the virus was not necessary, and all strains tested produced identical reactions.

Virus Titrations.—Bone marrow and buffy coat cultures have been compared in the titration of ASF virus and are equally

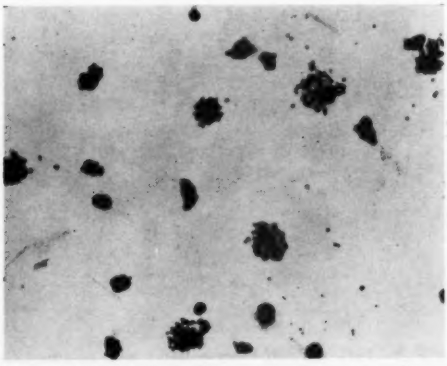

Fig. 4—Infected buffy coat culture, 72 hours postinoculation, showing cytopathic effect and hemadsorption. Leishman's stain; x 133.

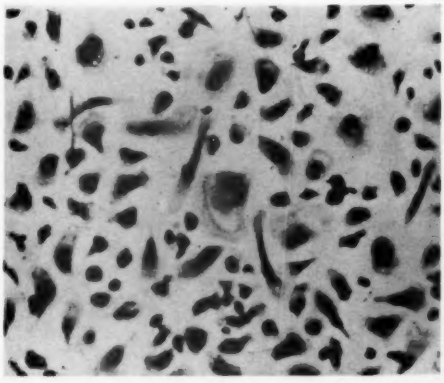

Fig. 5—Noninfected, 72-hour buffy coat culture. Leishman's stain; x 133.

suitable. However, the use of buffy coat cell cultures is preferred since it is simpler and does not involve killing the animal. Infectious fluids derived from cultures of ASF virus in suspended spleen fragments, bone marrow, or other buffy coat cultures have been titrated. Material from positive tubes produced signs of ASF when inoculated into pigs, whereas fluid from cultures determined to be negative after prolonged observation did not.

Virus-Neutralization Studies.—The results of infecting susceptible cells with ASF virus, as mentioned before, involves two reactions, *i.e.*, hemadsorption followed by cytolysis. The addition of serum from survivor-carriers inhibited the hemadsorption reaction, but did not significantly alter the cytopathic effect. Therefore, in testing blood or serum from a survivor, it is possible to observe a cytopathic effect without hemadsorption. Subinoculation from a tube showing only cytopathic effect will produce hemadsorption followed by cytolysis.

DISCUSSION

The susceptibility of certain types of cells in bone marrow and buffy coat cultures to the action of ASF virus appears to be specific and should serve to differentiate ASF and hog cholera viruses. The marked predilection of ASF virus for cells of the reticuloendothelial system and lymphocytic cells observed by some authors [2,9] serves to emphasize this point. On the other hand, Dunne *et al.*[5] reported that cultures of swine leukocytes are not affected by hog cholera virus. Furthermore, hog cholera virus did not produce hemadsorption or cytolysis in bone marrow cultures prepared in the same way as those used in our investigations.*

In addition to the apparent specificity of the reaction, cultures of buffy coat cells are easily prepared and are susceptible to ASF virus without preliminary adaptation. These properties, together with the fact that as little as 0.05 ml. of blood from a pig in the acute phase of ASF will often produce positive results within 24 hours, make it a valuable diagnostic aid. Erythrocytes are an important part of the test, and the concentration in the culture system appears to be unimportant, provided it does not interfere with attachment and survival of cells. However, since relatively few erythrocytes are needed as indicators, the concentration used has been the minimum consistent with methods of harvesting the buffy coat.

The term "hemadorption" has been used to describe the phenomenon of erythrocytes adhering to the surface of infected cells, since presumably the mechanism is similar to that described by Vogel and Shelokov [15] and Shelokov *et al.*[13] However, it has not been possible to demonstrate hemagglutinin in supernatant fluid of infected tissue cultures or in serum and tissue extracts of infected animals. Presumably, hemagglutinin is not present in quantities sufficient to bring about hemagglutination in the test tube, or the proper procedures have not been used. From preliminary observations, it appears that the hemagglutinin is distinct from the infectious particle, since survivor-carrier serum containing ASF virus will inhibit the hemadosorption reaction but will not neutralize the cytopathic effect. Whether the hemagglutinin is identical in all strains of ASF virus at our disposal will be the objective of future investigation.

SUMMARY

1) Swine bone marrow and buffy coat cells were cultured on a glass substrate, using 80 per cent mixture 199 and 20 per cent normal pig serum as nutrient medium.

2) African swine fever (ASF) virus infected certain large granular cells (macrophages) and produced two distinct reactions—hemadsorption followed by cytolysis. Fibroblast-like cells were not involved in the reaction and continued to proliferate in infected cultures.

3) It is believed that the reactions observed are specific for differentiation of ASF and hog cholera viruses.

4) Several different sources of ASF virus were used as inoculum; adaptation was not necessary. All of the strains studied thus far produced identical reactions.

5) The time required for the appearance of a hemadsorption reaction depended upon the titer of the virus. As little as 0.05 ml. of infectious blood collected during the acute phase gave positive results in 24 hours or less.

6) The results of titration of material containing ASF virus were comparable in bone marrow and buffy coat cell cultures;

* Work with hog cholera virus was done by Dr. C. N. Dale and Mr. J. R. Songer, Animal Disease Station, Beltsville, Md.

however, the latter were preferred as they were easier to prepare.

7) Serum from survivor-carriers inhibited the hemadsorption reaction but did not neutralize the cytopathic effect to a significant degree. It is believed that the antigen responsible for hemadsorption is distinct from the infectious particle.

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Lack of Larvacidal Action of Ronnel and Bayer 21/199 Against Migrating *Ascaris suum* in Baby Pigs

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TWO ORGANIC PHOSPHORUS COMPOUNDS, ronnel (0,0-dimethyl 0-[2, 4, 5-trichlorophenyl] phosphorothioate)* and Bayer 21/199 (0,0-diethyl 0-[3-chloro-4-methyl-7-coumarinyl] phosphorothioate),† have been widely applied during the last few years as systemic larvacides for grubs in cattle. In addition to their insecticidal properties, several investigators have reported that these compounds have anthelmintic activity, primarily against trichostrongyle parasites of ruminants.[1, 3, 4, 5]

When used as grubicides, these organic phosphates exert their insecticidal function while in the circulatory system of the host and, for this reason, are referred to as systemic insecticides. Compounds with this property are essential for killing migrating larvae of *Ascaris suum*. The above compounds were fed to baby pigs during the early stages of Ascaris infection to determine whether they would kill the migrating larvae.

MATERIALS AND METHODS

Fourteen disease-free pigs, about 2 weeks old and weighing approximately 2 kg. each, were placed together in a concrete-floored room. Each was fed 1 pint of milk daily in addition to a pre-weaning ration fed ad libitum.

Each pig was given approximately 50,000 decoated eggs of *A. suum* by gavage. Five of the pigs were given 12.5 mg. of Bayer 21/199 per kilogram of body weight and 5 were given 50 mg. of ronnel per kilogram of body weight. The remaining 4 pigs served as untreated controls. The drugs were administered in gelatin capsules before the morning feeding for five consecutive days.

Blood samples were taken from all of the pigs before treatment and when killed for necropsy five days later. Whole blood cholinesterase determinations were made by the method of Biggs *et al.*[2] The livers and lungs were examined for larvae by microscopic observation of suspensions of the ground organs.[6]

RESULTS

Neither treatment significantly reduced the number of migrating larvae. An average of 1,728 larvae was found in the pigs treated with Bayer 21/199, 7,411 in those treated with ronnel, and 2,534 in the controls.

Two of the 5 pigs treated with Bayer 21/199 died of toxicosis. The first appeared lame at 1:30 p.m. on the second day of the trial, after a total dosage of 50 mg. of Bayer 21/199. It was isolated, and when observed at 4:30 p.m. it could get up with some difficulty but was unable to walk in a straight line. When the pig tried to eat the evening ration it collapsed and about 10 ml. of saliva was secreted onto the floor of the cage during 30 minutes of observation. The pig remained prostrate and died at 10:00 a.m. the following morning. The second pig died during the night of the fifth day, after a total dosage of 125 mg. of Bayer 21/199. No overt signs of toxicosis were evident in the 5 pigs treated with ronnel.

Since the organic phosphates are known to be potent cholinesterase inhibitors, the activity of the enzyme was determined in blood samples both before and after treatment. The results of these analyses are summarized (table 1).

The Bayer 21/199 reduced cholinesterase levels 93 per cent or almost completely, while a dosage of ronnel approximately four times larger reduced the levels approximately 60 per cent.

TABLE 1—Whole Blood Cholinesterase Activity of Pigs Before and After Treatment with Organic Phosphates

Compound	Dosage (mg./kg./day)	Cholinesterase units* Pre-treatment	Cholinesterase units* Post-treatment
Bayer 21/199	12.5	66	5
Ronnel	50.0	61	24
None	.....	71	64

* Units are micromoles of acetic acid formed per hour per milliliter of blood.

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* Ronnel is the name approved by the Committee on Insecticide Terminology of the Entomological Society of America. This compound has been sold as Trolene, Et-57, Korlan, and Et-14 by Dow Chemical Co., Midland, Mich.

† Bayer 21/199 is available from Chemagro Corp., New York, N.Y.

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DISCUSSION

Although these two organic phosphates (ronnel and Bayer 21/199) are effective as grubicides and anthelmintics against intestinal nematodes of ruminants, they are ineffective against the migrating larvae of *A. suum*, probably because the toxic properties of these compounds indirectly limit their systemic concentration. Herlich and Johnson [4] gave ronnel to cattle at the rate of 100 mg. per kilogram of body weight; since the pig has a simple stomach, the drug level was reduced by one half. A reduction of blood cholinesterase was still observed.

Bayer 21/199 has been administered to sheep and cattle at 25 mg. per kilogram of body weight.[5] For pigs in the experiments, the level of medication with this drug was reduced by one half. Even this level produced deaths. It appears that the systemic level which is toxic to swine is below the effective anthelmintic level of these organic phosphates.

SUMMARY

Daily doses of ronnel (0,0-dimethyl 0-2,4,5 [trichlorophenyl] phosphorothioate) at 50.0 mg. per kilogram of body weight and Bayer 21/199 (0,0-diethyl 0-[3-chloro-4-methyl-7-coumarinyl] phosphorothioate) at 12.5 mg. per kilogram of body weight had no effect on the migratory stage of *A. suum* in pigs 2 weeks old.

Both drugs elicited toxic effects over the five-day trial period, as shown by the reduction of whole blood cholinesterase and the death of 2 pigs given Bayer 21/199.

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The Canine Distemper-Measles Complex. I. Immune Response of Dogs to Canine Distemper and Measles Viruses

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THE POSSIBILITY that man might become infected with canine distemper virus (CDV), first suggested by Bryan [5] and Nicolle [20] and subsequently by Pinkerton *et al.*,[21] has, within the past six years, been approached experimentally. The adaptation of CDV, first to embryonating eggs, by Haig [16] and Cabasso and Cox,[6] and later to suckling mice, by Morse [19] and Carlström,[9] together with the simple cultivation of measles virus in tissue culture by Enders and Peebles,[12] provided a complete set of diagnostic tools for investigating the problem beyond the earlier observations of histopathologic and clinical similarities.

Adams [1] has presented clinical and serologic findings supporting the thesis that CDV is the specific cause of an acute respiratory illness of children. Subsequently, Adams, Imagawa, Yoshimori, and Huntington [2] described similarities in the inclusion bodies and giant cells found in CDV- and measles-infected tissues. An immunologic relationship was claimed on the basis of cross-neutralization demonstrable in tissue culture.

Later, Adams and Imagawa [3] reported both the neutralization of measles virus by anti-canine distemper serum prepared in ferrets, and the protection of mice against mouse-adapted distemper by measles antibodies produced in ferrets inoculated with this virus and adjuvant. Additional sources supporting a common measles-CDV antigen are Karzon's finding of CDV-neutralizing antibodies in almost 100 per cent of human adults [17] and Carlström's [10] observing a high correlation between a history of measles in children and the presence in their serums of heat-stable substances which would neutralize CDV. Through the successful adaptation of measles virus to suckling mice, Carlström succeeded in making definitive studies of cross-neutralization between distemper and measles in the same host system.[8]

The marked susceptibility of canine tissues to measles virus cultivated *in vitro* [13] suggested to the authors a study of the canine distemper-measles complex in a canine host. Accordingly, we have investigated the antibody response of dogs to living and inactivated canine distemper or measles virus and the protection against several strains of canine distemper virus provided by such antigenic exposure. Our findings, in addition to substantiating the existence of an antigenic relationship between strains of CDV and measles, also suggests the possibility of an antigenic mosaic or gradient with certain distemper strains being more closely related to measles virus than are others.*

MATERIALS AND METHODS

Dogs.—Litters of mixed breed pups, farm or home raised, were purchased locally. Depending upon the design of the experiment, dogs 4 to 12 weeks of age were caged as litters or individually in comfortable, warm, isolated quarters without cross-ventilation. This area was tested at intervals for the absence of latent canine distemper by placing a live susceptible sentinel ferret in proximity to the dogs. Temperatures of all dogs were taken daily and weights recorded where pertinent. Blood samples were taken from all animals by cardiac or femoral artery puncture under anesthesia. At the time of CDV challenges, dogs were removed to a second set of exposure quarters.

Viruses.—All viruses were stored before use at −60 C. in dry ice. The following strains of canine distemper virus were employed:

Strain A—"Wisconsin," [11] a "wild" strain isolated from a racecoon, was used in the second raccoon passage as a 20 per cent spleen suspension with a titer of $10^{5.3}$ e.i.d.$_{50}$ per milliliter as measured in egg chorioallantoic membranes (CAM).

Strain B—Onderstepoort,[18] egg-adapted from ferret, was received as ferret spleen suspension and had six ferret plus two egg-CAM passages before use in the current study as a 20 per cent chicken embryo tissue suspension with a titer of $10^{4.0}$ e.i.d.$_{50}$/ml.

Strain C—Onderstepoort, maintained exclusively in ferret spleen, was used as a 10 per cent ferret spleen suspension, $10^{6.3}$ i.d.$_{50}$/ml. as titrated in ferrets.

Strain D—egg-adapted,[6] commercial canine distemper vaccine** at about 49th egg passage, was

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** This vaccine was produced by Lederle Laboratories, Pearl River, N.Y.

passed through ferrets 12 times and on the egg allantoic membrane for three additional passages. It was used as 10 per cent CAM suspension titering 10^5 e.i.d.$_{50}$/ml.

Strain E—same as strain D but at the 12th ferret passage and used as 10 per cent ferret spleen suspension. The titer is unknown but estimated to be between $10^{4.0}$ and $10^{6.0}$ i.d.$_{50}$/ml. for the ferret.

Strain F—egg-adapted commercial canine distemper vaccine at the 29th egg chorioallantoic membrane passage, used as 20 per cent CAM suspension with a titer of $10^{5.0}$ e.i.d.$_{50}$/ml.

Strain G—Virulent distemper challenge virus (Snyder Hill strain) passed four times in dog spleen and used as a 20 per cent or 50 per cent suspension of dog spleen or 10 per cent suspension of canine brain. Expected titer of such materials is about $10^{4.0}$ to $10^{5.0}$/ml. in ferrets.

Measles Virus.—The strain used was the Edmonston[12] grown in human heart tissue culture[15] with t.c.d.$_{50}$/ml. ranging between $10^{5.5}$ or $10^{6.2}$ or monkey kidney tissue culture with a t.c.d.$_{50}$/ml. of $10^{5.2}$. Inactivated measles vaccines were prepared from each of these sources by the addition of 1:4,000 formaldehyde followed by incubation of the virus for five to six days at 37 C. Subsequent safety tests in human heart cell cultures were negative for residual live virus.

EXPERIMENTAL PROCEDURES

Distemper.—Egg-adapted CDV was titrated and neutralization tests were performed on the CAM of 7- to 9-day-old chicken embryos. For neutralization tests, equal parts of virus and dilutions of dog serums inactivated at 56 C. for 15 minutes, were incubated for two hours at 4 C. and 0.2 ml. of each mixture was then inoculated on the CAM of at least five eggs. After six to seven days' incubation, membranes were removed and the characteristic distemper lesions counted. Two different doses of challenge virus were used in these tests. Preliminary experiments (tables 1 and 2) employed approximately 100 e.i.d.$_{50}$ of virus, whereas in subsequent work the amount was increased to between 300 and 1,000 e.i.d.$_{50}$. In any one experiment, the pre- and postvaccination blood tests were run simultaneously and titered against a given virus concentration. Most prevaccination serums were tested in twofold dilution from 1:2; postvaccination from 1:50. Neutralization titer is expressed as the calculated serum dilution (using the Reed-Muench accumulation) which protected against 50 per cent of focal lesions on the egg membrane.

Non-egg adapted strains of canine distemper virus were titrated in ferrets by intraperitoneal inoculation using 1 ferret for each dilution.

Measles Virus.—For vaccine and antigen, virus was grown in tissue culture in Eagle's basal medium using Earle's instead of Hank's balanced salt solution. The medium was used both with and without additional 3 per cent chicken serum added to prolong cell life. Neutralization tests were performed in HeLa cell roller tube cultures fed with medium 199. Each serum, inactivated at 56 C. for 30 minutes, plus approximately 100 t.c.d.$_{50}$ of virus was incubated for one to two hours at 4 to 6 C. and the mixtures then inoculated in each of five HeLa cell cultures. The tubes were observed for cytopathic effect up to 21 days, with the fluids being changed approximately each seven days where necessary.

Measles complement-fixation (CF) tests were performed as described previously,[15] using a modified Kolmer procedure with overnight fixation. Antigens consisted of measles-infected human heart or monkey kidney tissue culture fluids from 5- to 10-day-old cultures from which the cells were removed by low-speed centrifugation. Such antigens had an infective titer between $10^{4.0}$ and $10^{6.2}$ t.c.d.$_{50}$/ml. and a CF titer between 1:2 and 1:8 against convalescent human or monkey anti-measles serums. All antigens were appropriately diluted, so that approximately two to four units were employed. Controls of noninfected tissue culture fluids and positive and negative canine serums were included in each test. Two plus or greater fixation was regarded as positive.

RESULTS

Three general questions were investigated in the following series of experiments. These were: (1) Would dogs vaccinated with live canine distemper virus develop antibodies to measles? (2) Conversely, would inactivated measles virus produce measles antibodies or canine distemper antibodies in dogs? (3) What would be the clinical and immunologic response of dogs with or without previous exposure to canine distemper to infection with live measles virus?

RESPONSE OF DOGS TO LIVE CANINE DISTEMPER VACCINE

A series of three experiments was performed to determine the response of dogs to canine distemper vaccine when their antibody level was measured to both canine distemper virus and measles antigens.

In experiment 1, blood samples were taken from 6 dogs (3 each from 2 litters) which were then inoculated subcutaneously with 2.0 ml. of egg-adapted (strain F) CDV having an e.i.d.$_{50}$ of approximately $10^{5.0}$/ml. One month later, blood samples were again taken and the dogs were then challenged by the intracerebral inoculation of 0.25 ml. of the virulent Snyder Hill strain of CDV in the form of 20 per cent canine spleen suspension. All 6 animals survived, whereas 2 nonvaccinated control

animals succumbed on the 11th and 19th day, respectively. While only three of the six serums were available for prevaccination antibody determinations, these were negative for both agents (table 1).

Following immunization, a moderate rise in CDV-neutralizing antibodies appeared, but the measles CF titer was only minimal. However, following challenge, there was not only a considerable elevation of the CDV level with titers of 1:800 and 1:3,200, but this was also paralleled by a rise in the measles CF titer. It was of interest that the 3 dogs with highest postchallenge CDV antibody titers had a higher average level of measles CF antibodies.

Two additional experiments designed to compare levels of antibodies to CDV and measles resulting from CDV vaccination were carried out but, since they included other antigens as well, a discussion of these will be deferred until after a consideration of measles infection in dogs.

TABLE 1—Antibody to Canine Distemper and Measles Viruses in Dogs Following Administration of CD Vaccine and Subsequent Distemper Challenge

Dog No.	Prevaccination		30 days Postvaccination*		30 days Postchallenge**	
	CD neut. ab.	Measles CF	CD neut. ab.	Measles CF	CD neut. ab.	Measles CF
1015-1	<1:50	<1:8	1:100	1:8	1:3,200	1:32
1015-4	<1:50	<1:8	1:100	1:8	1:1,600	1:16
1015-5	<1:50	<1:8	1:100	1:8	1:1,600	1:32
1017-1	.....	.....	1:100	.....	1:800	1:16
1017-2	.....	.....	1:400	.....	1:800	1:8
1017-3	.....	.....	1:100	.....	1:800	1:8

* Initial vaccine was egg-adapted CDV, 2.0 ml. subcutaneously. ** Challenge was canine brain CDV, Snyder Hill strain, 0.25 ml. intracerebrally.

RESPONSE OF DOGS TO INACTIVATED MEASLES VIRUS

In each of two experiments, dogs were first vaccinated with measles virus, then challenged with living canine distemper virus. In the first study, 6 pups in two litters received a total dose of 1 ml., intraperitoneally, of formalin-inactivated virus grown in human heart tissue culture, vaccine A.

Canine distemper-neutralizing and measles CF antibody levels were absent or of minimal level prior to vaccination (table 2). Thirty days later, measles complement-fixing activity had increased to 1:64 in each of 5 dogs tested and 5 of 6 dogs had developed slight CDV antibody levels of 1:50 and 1:100. Each of these and 4 additional nonvaccinated controls from the same litters were then challenged intraperitoneally with 1.0 ml. of virulent Snyder Hill virus. Two of the 6 vaccinated and all of the 4 control animals succumbed

TABLE 2—Resistance and Antibody to Canine Distemper Following Vaccination of Dogs with Killed Measles Virus

Dog No.	Prevaccination		Vaccine	30 days Postvaccination		Challenge with CDV
	CD neut. ab.	Measles CF		CD neut. ab.	Measles CF	
804	1:4	1:16	Measles virus, (human heart tissue culture) inactivated with 1:4,000 HCHO.	<1:50	1:64	Survived
805	<1:4	<1:16		1:50	1:64	Died
806	1:4	1:32		1:50	1:64	Survived
807	<1:4	<1:16		1:50	Not done	Survived
1	1:16	1:16		1:100	1:64	Died
3	1:8	1:32		1:100	1:64	Survived
5	1:16	Not done	None, contact controls.	<1:50	Not done	Died
6	1:8	Not done		<1:50	Not done	Died
7	1:32	Not done		<1:50	Not done	Died
8	1:4	Not done		<1:50	Not done	Died

Challenge = a 20 per cent suspension of dog spleen, Snyder Hill strain, approximately 10^4 l.d.$_{50}$ in 1.0 ml. intraperitoneally.

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within 30 days, with characteristic signs of distemper, suggesting that only slight, if any, protection was conferred by a measles (killed virus) vaccine.

In this experiment, the pups were put on test shortly after procurement, and it was considered possible that the minimal antibodies observed might have been due to a canine distemper infection naturally acquired during the 30-day postvaccination period but modified by the presence of measles antigen or residual colostral immunity.

Since Gillespie *et al.*[14] have shown the development of distemper immunity in pups is dependent upon loss of colostral protection, it was decided to perform a second study of measles (killed virus) vaccine in which the pups immediately after purchase were given CDV protection by means of antiserum. This experiment was combined with the study of live measles and canine distemper virus response and will be described in a later section of this report.

RESPONSE OF DOGS TO INOCULATION OF LIVE MEASLES VIRUS

Inoculation of dogs with living Edmonston strain virus, in contrast to inactive measles antigens, produced a subclinical infection with the appearance of measles-neutralizing and CF antibodies and a lesser, but significant, degree of CD antibody formation and resistance. Three extensive experiments employing live measles virus were performed during the past 18 months. Because each was somewhat modified in design in order to obtain answers to specific problems which arose in the course of the study, they will be discussed separately.

Experiment 1.—Pups from five litters, between 5 and 7 weeks old, were allotted as shown (table 3). Dogs 808 through 829 were inoculated intramuscularly with 1 ml. of measles-infected human heart tissue culture containing $10^{5.8}$ t.c.d.$_{50}$/ml. Dogs 836–839 were fed with 1.0 ml. of this material, while dogs 840–842 remained as controls.

Base line serums available from 18 of these pups were found to be devoid of distemper-neutralizing antibodies at the time of infection. Measles antibody formation was not determined but presumed to be absent on the basis of a subsequent absence in the litter-mate feeding or control dogs 837, 839, 840, and 842. None of the animals inoculated with or fed measles virus developed any obvious signs of illness.

Two and three weeks after vaccination, serum samples were tested for measles antibodies and a three-week serum speci-

TABLE 3—Antibody to Canine Distemper and Measles Following Exposure of Dogs to Live Measles Virus*

Dog No.	Age of litters	Method of exposure	Prevaccination CD neut. ab.	Postvaccination: Measles CF 13 days	Postvaccination: Measles CF 21 days	Postvaccination: Measles Neut. ab. 13 days	Postvaccination: CDV Neut. ab. 21 days
808			1:8	1:32	1:16	1:16	1:200
809			1:8	1:32	1:16	1:16	1:50
810	5 weeks	1.0 ml.	1:32	1:32	<1:16	1:16	1:100
812		i.m.	.....	1:64	1:32	.....	1:200
813			1:8	1:32	<1:16	.....	.....
815			.....	1:16	1:16	.....	.....
816			<1:8	1:32	1:32	1:32	1:100
817			<1:8	1:32	1:16	1:16	1:50
818	6 weeks	1.0 ml.	.....	1:64	<1:16	1:64	.....
819		i.m.	1:8	1:64	1:64	1:64	1:50
820			.....	1:64	1:64	.....	.....
821			<1:8	1:64	1:128	.....	1:200
824			<1:8	1:16	1:32	1:16	1:100
825			<1:8	1:16	<1:8	1:8	1:100
826	7 weeks	1.0 ml.	<1:8	1:32	1:32	1:16	1:100
827		i.m.	<1:8	1:32	1:64	.....	1:100
828			<1:8	1:64	1:64	.....	1:50
829			.....	1:16	<1:16	.....	.....
836			1:16	.....	<1:16	<1:8	<1:50
837	6 weeks	1.0 ml.	1:32	<1:16	<1:16	<1:8	<1:50
839		per os	<1:8	<1:16	<1:16	.....	<1:50
840		None	1:8	<1:16	<1:16	<1:8	<1:50
842	7 weeks	controls	1:16	<1:16	<1:16	....	<1:50

* Infection = $10^{5.8}$ t.c.d.$_{50}$ of human heart tissue culture virus per 1.0-ml. dose.

TABLE 4—Response of Litter Mates to Several Strains of Canine Distemper and Measles Virus

Virus	Strain	Dog No.	Litter No.	Preinfection		Postinfection 10 days		Postinfection 20 days		CDV challenge*
				CD	Measles CF	CD	Measles CF	CD	Measles CF	
Measles	Edmonston	194	39	<1:10	<1:16	<1:40	1:128	1:150	1:64	Survived
		195	39	<1:10	<1:16	<1:40	1:128	1:100	1:128	Survived
		200	40	<1:10	<1:16	<1:40	1:128	1:100	1:128	Survived
		208	41	<1:10	<1:16	<1:40	1:128	1:100	1:64	Survived
		209	41	<1:10	<1:16	<1:100	1:32	1:100	1:32	Dead—14 days
		210	41	<1:10	<1:16	<1:40	1:32	<1:100	1:64	Survived
		217	41	<1:10	<1:16	<1:40	1:64	<1:100	1:64	Survived
CDV	A	201	40	<1:10	<1:16	1:240	1:16	1:800	1:32	Survived
	A	211	41	<1:10	<1:16	1:240	1:16	>1:800	1:16	Survived
	B	202	40	<1:10	<1:16	1:60	<1:16	1:100	>1:16	Survived
	B	212	41	<1:10	<1:16	<1:40	<1:16	1:200	<1:16	Survived
	C	203	40	<1:10	<1:16	1:40	1:16	1:600	1:64	Survived
	C	213	41	<1:10	<1:16	1:240	1:16	>1:800	1:32	Survived
	D	204	40	<1:10	<1:16	<1:40	<1:16	<1:100	<1:16	Survived
	D	214	41	<1:10	<1:16	1:40	<1:16	<1:100	<1:16	Survived
	E	205	40	<1:10	<1:16	1:60	<1:16	<1:100	1:16	Survived
	E	215	41	<1:10	<1:16	Not done	<1:16	<1:100	Not done	Survived
	F	206	40	<1:10	<1:16	1:80	1:128	1:150	>1:128	Survived
	F	216	41	<1:10	<1:16	<1:40	<1:16	1:600	<1:16	Survived
None controls		196	39	NOT TESTED						Dead—16 days
		197	39							
		207	40							Dead—14 days
		218	41							Dead—16 days

* 2.0 ml. (intraperitoneally) suspension of 50 per cent dog spleen.

men was tested for CD antibodies. Measles titers ranging from 1:16 to 1:64 appeared within 13 days in the first three litters (Table 3). These levels were retained or diminishing by the third week. A low measles-neutralizing antibody titer closely paralleling the complement-fixing antibody titer was also observed in the blood samples obtained from this group after two weeks. Finally, inoculation of live measles virus resulted in the concomitant development of a moderate level of distemper-neutralizing antibodies in these animals.

In contrast, the 3 pups which were fed live measles virus and the 2 control dogs failed to manifest any change in their serologic picture. In this experiment, the immunity of the dogs was not challenged at the conclusion of the experiment.

Experiment 2.—This was performed approximately eight months later and differed from experiment 1 in several important respects. Both live measles and several strains of canine distemper virus were employed and resistance to CD was also tested eight weeks after the initial exposure to either virus. Measles infection was accomplished by the intraperitoneal inoculation of $10^{4.5}$ t.e.d.$_{50}$/ml. of virus grown in human heart tissue culture. This was given to 7 pups 6 to 8 weeks of age and from three different litters. For canine distemper, a second group of 12 animals was divided into pairs and each pair vaccinated with a different strain of CDV (table 4). Preinfection serums were uniformly devoid of CD-neutralizing or measles CF antibodies.

The dogs infected with live measles virus responded as did those in experiment 1 (table 3); that, is, without overt disease but with the appearance of both measles and CD antibodies, the latter being present only at low to moderate levels. It should be emphasized that neutralization tests performed on these serums employed a virus dose of approximately 1,000 e.i.d.$_{50}$ of virus, whereas the tests performed in experiment 3 employed approximately 300 e.i.d.$_{50}$ of virus. This will account for the discrepancy in the level of CD-neutralizing antibodies which appeared between these two studies.

The dogs inoculated with distemper virus responded in a manner which depended upon the strain of virus employed. Strains A, C, and F elicited neutralizing antibody titers of between 1:150 and 1:800 and all animals but one, dog 216, developed measles CF activity. Infection with strain B elicited distemper antibody titers of only 1:100 and 1:200 and an equivocal measles CF, whereas strains D and E failed to stimulate a response to either virus. Both

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strains D and E were live viruses recently passaged in ferrets. Also, all tests were performed using strain F, egg-adapted virus, as the test strain in the egg system.

The immunity of all of these dogs was challenged intraperitoneally with virulent (strain G) distemper virus six weeks after the start of this experiment. Only 1 dog, 209, in the measles group, succumbed to infection, which was fatal to each of 3 control pups. Although no virus could be isolated from dog 209 by ferret passage, death must be presumed to be due to distemper because of the signs developed and its concurrence with deaths in the control group.

Experiment 3.—In both previous experiments, designed to compare the immunologic response to measles and to canine distemper, pups were infected or vaccinated at a relatively early age and were presumed to be free of latent distemper on the basis of absence of antibodies in their prevaccination serums. However, because the possibility existed, albeit a remote one, that the appearance of distemper antibodies in dogs inoculated with live measles virus was due to a parallel naturally acquired distemper infection, a third study was performed using pups passively immunized with anti-canine distemper serum. As in experiment 2, a determination of antibody patterns following distemper challenge was also included in this series.

Twenty-eight weanling pups, 8 weeks old, were subcutaneously inoculated with commercial antiserum** using 1.0 ml./lb. Eight weeks later, when this serum level was presumably eliminated, blood samples from the dogs were found to be free of distemper or measles antibodies. They were then inoculated with 1 ml. subcutaneously as follows:

8 dogs (No. 1–8) were given egg-adapted live canine distemper vaccine.†

7 dogs (9–15) were given measles vaccine prepared from monkey kidney tissue culture as described above.

7 dogs (16–22) were inoculated with live measles virus in human heart cell culture; this lot had a titer at harvest of $10^{4.5}$ t.c.d.$_{50}$/ ml.

4 dogs (24–28) were left untreated as contact controls.

Blood samples were taken from all animals two, four, six, and eight weeks after exposure to virus, during which period they remained uniformly afebrile and healthy. At the end of eight weeks, the immunity of all 28 dogs was challenged with virulent Snyder Hill distemper virus by the intravenous inoculation of 1 ml. of fresh 10 per cent spleen suspension.

Following the challenge, daily temperatures and weights of all dogs were obtained for a six-week period. Although several dogs in the control and vaccinated groups had transient fever and some were acutely ill, none succumbed to this challenge. (Dogs 25 and 26 in the control group were removed from the study for other purposes prior to the challenge.)

The antibody pattern presented in this series of animals is summarized (table 5). Neither inactivated measles nor live attenuated distemper vaccine provoked the appearance of measles antibodies.

These findings are at variance with those shown in table 2, where a slight rise in CDV and measles antibodies resulted from vaccination with measles (killed virus) vaccine. The discrepancy is difficult to explain. However, it may be accounted for by the fact that a different, and perhaps more potent, lot of vaccine was used in the first study and by the pretreatment of dogs with anti-canine distemper serum in the experiment tabulated in table 5. Of interest is the finding that prior exposure to inactivated measles antigen caused a significant alteration in the postchallenge serologic response. Whereas the pooled serums of 4 control, nonvaccinated dogs had a CDV-neutralization titer of 1:140 and 1:2,240 at two and four weeks postchallenge, respectively, those animals which had experienced a previous contact with measles antigen had titers of 1:1,120 and 1:3,170. More striking, perhaps, were the increases in the measles CF level.

We conclude from this experiment and from the data on dogs illustrated in table 2 that a single dose of an inactivated measles vaccine, while eliciting little or no previous primary antibodies to itself or to distemper, nevertheless sensitized the dogs so that a subsequent challenge with virulent canine distemper virus provoked a level of serologic response having the characteristics of an anamnestic reaction.

As stated previously, measles CF antibodies appeared rapidly in response to live measles virus but had begun to wane by the eighth week. Comparing distemper and measles viruses as antigens, CD neutralization with levels of 1:500 appeared within two weeks after distemper vaccination and continued to increase to 1:2,360 at eight weeks. Lower, but significant, titers of distemper antibody were induced by inoculation of live measles virus. Following the intravenous inoculation of virulent distemper-infected dog spleen into dogs of

** This antiserum was produced by Fort Dodge Laboratories, Fort Dodge, Iowa.

† This vaccine was a product of Anchor Serum Co.,

these groups, there was a dramatic appearance of measles CF antibodies in all animals with, as might have been anticipated, highest titers observed in the groups previously infected with live measles.

RESPONSE OF THE FERRET, CAT, AND RABBIT TO LIVE MEASLES VIRUS

Is measles virus able to produce subclinical infection in other species besides man, monkey, and dog? Is susceptibility to distemper commonly associated with susceptibility to measles virus? An experiment designed to answer these questions was carried out with the ferret, a species highly susceptible to distemper; the cat, which is resistant to distemper; and the rabbit.

Blood samples were obtained from 2 ferrets, 4 cats, and 4 rabbits; these animals were then inoculated subcutaneously with 1.0 ml. of live measles virus in human heart tissue culture, titer $10^{5.6}$ t.c.d.$_{50}$/ml. Ten and 20 days later, these animals were again tested for the development of complement-fixing antibodies for measles and all were found negative. Several rabbit serums reacted with measles antigen to titers of 1:16 to 1:32 (table 6), but this was unrelated to the infection and, we suspect, represents a nonspecific reaction in the species. The ferrets were also tested for any evidence of induced resistance to canine distemper by an intranasal challenge of 1.0 ml. of a 10 per cent suspension of canine distemper-infected ferret spleen. Both animals died from typical canine distemper. This experiment, although involving few animals, suggests that the dog may be rather unique in its susceptibility to measles virus infection. More work along these lines is in progress.

DISCUSSION

Because these findings are somewhat complex, a brief recapitulation may be desirable.

a) Although live egg-adapted CD vaccine caused only a slight or moderate rise in measles antibody, when CD-vaccinated dogs were challenged with virulent CD virus an elevation of both CDV and measles antibody resulted. Strain and passage of the CD virus used may be a determining factor in the response to the distemper antigen.

b) Inoculation of dogs with killed measles virus failed to elicit measles or CD anti-

TABLE 5—Response of Dogs Passively Immunized to CDV and Subsequently Vaccinated with CDV, Living or Inactivated Measles Virus

Dog No.	Vaccine 1.0 ml.s.c.	Pre-inoc.	Measles CF antibody titers** Weeks post-Immunization 2	4	6	8	CDV challenge 2	4	Distemper neutralizing antibody titers** Weeks post-Immunization 2	4	6	8	CDV challenge 2	4
							Measles							
1		<8	<8	<8	<8	<8	<32	<32						
2		<8	<8	<8	<8	<8	<32	<32						
3	Canine	<8	<8	<8	<8	<8	<32	<32						
4	distemper	<8	<8	<8	<8	<8	<32	<32	500*	2,760	2,200	2,360	3,550	3,990
5	(live, avian)	<8	<8	<8	<8	<8	<32	<32						
6		<8	<8	<8	<8	<8	<32	<32						
7		<8	<8	<8	<8	<8	<32	<32						
8		<8	<8	<8	<8	<8	<32	<32						
9		<8	<8	<8	<8	<8	32	32						
10	Measles	<8	<8	<8	<8	<8	<32	32						
11	HCHO in-	<8	<8	<8	<8	<8	<32	32						
12	activated	<8	<8	<8	<8	<8	<32	<32	<20	<50	<20	40	1,120	3,170
13	(monkey	<8	<8	<8	<8	<8	32	32						
14	kidney t.c.)	<8	<8	<8	<8	<8	<32	32						
15		<8	<8	<8	<8	<8	....	....						
16		<8	>64	32	<8	16	256	128						
17		<8	32	32	16	16	>512	256						
18	Measles,	<8	>64	64	16	16	64	64						
19	live (human	<8	16	16	8	8	32	<32	<20	106	200	204	1,260	6,310
20	heart t.c.)	<8	32	32	NA	8	256	128						
21		<8	16	16	<8	<8	>512	256						
22		<8	>64	64	16	16	>512	.....						
24		<8	<8	<8	<8	<8	....	<32						
25	Controls	<8	<8	<8	....	....	....	....	<20	<50	<20		141	2,240
26		<8	<8	<8	....	....	....	....						
28		<8	<8	<8	<8	<8	<32	32						

* Arithmetic mean titer of pooled serums. ** Expressed as reciprocals of the serum dilution.

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bodies but, nevertheless, appeared to sensitize these animals so that when exposed to distemper, they responded as would hyperimmune animals to both viruses.

c) Dogs infected with the Edmonston strain of measles virus uniformly develop a subclinical infection with a subsequent appearance of both measles and distemper antibodies. Measles-convalescent dogs were found to be resistant to virulent distemper challenge.

d) Another species, the ferret, which is fully susceptible to canine distemper, proved resistant to measles in one limited experiment.

Judging from the studies reported here, together with the earlier investigations of Carlström,[8] Karzon,[17] Adams *et al.*,[2] there no longer seems to be adequate grounds for delaying the postulation of a group of viruses which includes measles and canine distemper. It is not unlikely that rinderpest will also be found to belong in this family as the evidence for its antigenic relation to distemper virus is more than superficial.[22]

The degree of antigenic overlap between measles and CDV requires additional quantitative immunologic investigation, but enough evidence is at hand to suggest that measles is the broader or "prime" antigen of the pair. Carlström[10] observed that homologous measles serum titers were greater by a ratio of about 5:1 when measles and CDV antibody levels were assayed by cross-neutralization in suckling mice. While our own findings are only preliminary, the fact that distemper-vaccinated dogs vary in their measles antibody response leads us to believe that it is premature to establish the degree of overlap based upon differential antibody patterns.

Additional evidence for variation in the characteristics in distemper strains are found in Rockborn's observations that two Swedish strains of CDV which were immunologically indistinguishable from an egg-adapted strain (Lederle) nevertheless produced only low antibody titers, and then after a considerably longer interval than did the Lederle virus.[23]

Intrinsic antigenic differences in CDV lines have also been uncovered in experiments in our own laboratory in which the serums from dogs vaccinated with a distemperoid strain of virus gave higher CF titers with measles antigen than serums from dogs vaccinated with other CDV strains.[18] Heterogeneity among measles strains also seems likely in view of the development of attenuated variants[12] and strains of slow and fast cytopathic effect in dog kidney tissue culture.[13]

Dogs infected with egg-adapted distemper virus developed no or minimal antibodies to measles, whereas infection of dogs with measles provoked CDV-neutralizing antibodies of respectable (± 1:200) levels. Superinfection of a measles-immune dog with CDV not only boosts the CDV antibody level but measles CF as well (*see* table 5). It is of interest that although inactivated measles virus failed to stimulate either measles or CDV antibodies in dogs, this antigenic exposure apparently served to sensitize the animals so that after a challenge with live CDV the distemper-neutralizing antibody elevation was accelerated (dogs 9–15, table 5).

Although the experiments described here suggest that measles infection of dogs will

TABLE 6—Response of Ferrets, Cats, and Rabbits to Inoculation of Live Measles Virus

Animal No.	Preinoculation Measles CF	Measles CF Postinoculation 10 days	Measles CF Postinoculation 20 days	CDV challenge
Ferret 223	<1:16	<1:16	<1:16	Died— 9 days
Ferret 224	Not done	<1:16	<1:16	Died—14 days
Cat 227	<1:16	<1:16	<1:16	
Cat 228	<1:16	1:16	<1:16	Not done
Cat 229	<1:16	<1:16	Not done	
Cat 230	<1:16	<1:16	<1:16	
Rabbit 231	1:32	1:32	AC	
Rabbit 232	<1:16	1:32	1:32	Not done
Rabbit 233	1:16	1:32	1:32	
Rabbit 234	<1:16	1:32	1:16	

AC = anticomplementary.

confer some protection against canine distemper, these studies were performed with "laboratory" strains of CDV, and we will require additional natural exposure experiments to better answer this important question. It is encouraging, however, that the CDV antibody levels which result from measles infection are the same order of magnitude as reported by Gillespie *et al.*[14] to be protective in pups.

The question of how closely related are the viruses of measles and distemper is an intriguing one. The serologic and immunologic cross relationship have already been referred to. Both agents propagate in either embryonating eggs or chicken cell culture,[7,12] and dog kidney tissue culture[13] or in suckling mice.[9] Both produce a viremia, an apparently similar inclusion body, are relatively thermolabile viruses, and are of similar particle size.

On the other hand, as evidence of their dissimilarity is the resistance of the ferret to measles infection and the failure of measles viral antigen to fix complement with potent CDV-convalescent serums. It may be pertinent to mention also the apparent lack of pathogenicity of egg-adapted CDV for man.[4] Certain broader questions, such as (1) whether measles infection in man is associated with CD in domestic hosts, and (2) whether CDV vaccine will confer significant protection against measles, are in the forefront of other continuing investigations at the present time.

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The Interrelationship of Diabetes Mellitus, Obesity, and Pyometra in the Dog

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OBESITY AND diabetes mellitus are frequently encountered simultaneously in both man and animals. Woodyatt [10] stated that about 70 per cent of human diabetic patients are obese. No conclusive statistical investigations have been found in the available literature.

Dogs affected with diabetes mellitus, obesity, and pyometra (chronic purulent endometritis) commonly present a similar clinical history and pathologic-anatomic picture. The present study was undertaken to investigate the relationship among these three diseases.

MATERIALS AND METHODS

From a total of 10,993 canine necropsies performed and analyzed at the Department of Pathology, Royal Veterinary College, Stockholm, during the period 1930 to 1957, there were 971 cases of obesity, 487 cases of pyometra, and 167 cases of diabetes mellitus.

The diagnosis, diabetes mellitus, was based primarily on the presence of hyperglycemia and glycosuria. When blood and urine sugar were not determined prior to death, qualitative glucose tests were performed on bladder urine at necropsy. In a few cases, diabetes mellitus was diagnosed on the basis of the classic morphological changes in the pancreatic islets—degranulation of β-cells. These rigorous requirements may have excluded some bona fide cases of diabetes mellitus because of a combination of no antemortem glucose determination, lack of urine at necropsy, and autolytic changes in the pancreatic islets.

The diagnosis of pyometra was readily determined. Dogs with pyometra, surgically treated some time before necropsy, were also included. Dogs with cystic endometritis, a condition possibly related to pyometra, were not included.

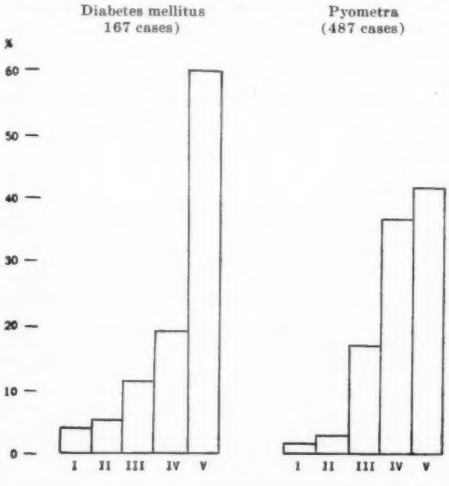
The obesity group included those dogs considered to be overly fat without coexisting diabetes mellitus or pyometra.

Five cases of coexisting diabetes mellitus and pyometra were encountered in the diabetes mellitus group. In some cases of pyometra, a coexisting diabetes mellitus may have been overlooked. Therefore, an accurate analysis of the frequency of diabetes mellitus in cases of pyometra cannot be made on the basis of the necropsy protocols.

Obesity was frequently found in association with diabetes mellitus and pyometra (fig. 1), but no double registration was made in these cases. The obese group included only examples of pathologic

TABLE 1—Average Ages of Dogs with Diabetes Mellitus, Obesity, and Pyometra

Condition	Age in years $\bar{x} \pm$ s.e.m.
1. Obesity	7.86 ± 0.12 yr.
2. Diabetes mellitus	8.03 ± 0.19 yr.
3. Pyometra	8.44 ± 0.12 yr.
"t" test:	
1.–2. Difference:	0.17 ± 0.22; t = 0.77
1.–3. Difference:	0.58 ± 0.17; t = 3.41***
2.–3. Difference:	0.41 ± 0.22; t = 1.86



	Diabetes mellitus % ± e	Pyometra % ± e
I Cachectic	4.2 ± 1.56	1.8 ± 0.60
II Below normal	5.4 ± 1.75	2.9 ± 0.77
III Normal	11.4 ± 2.47	17.0 ± 1.70
IV Good	19.2 ± 3.05	36.8 ± 2.19
V Obese	59.9 ± 3.80	41.5 ± 2.25

Fig. 1—The nutritional condition of dogs with diabetes mellitus and those with pyometra.

From the Department of Pathology (Krook), and the Department of Physiology (Larsson), Royal Veterinary College, Stockholm, Sweden; and from the Biology Department (Rooney), Virginia Polytechnic Institute, Blacksburg.

TABLE 2—Sex Incidence of Dogs with Diabetes Mellitus and Obesity

a. Diabetes mellitus	Male	Female
Case material	29	138
General dog population	3040	2898
$\chi^2 = 74.79^{***}$ (degrees of freedom = 1)		
b. Obesity	Male	Female
Case material	367	604
General dog population	3040	2898
$\chi^2 = 59.92^{***}$ (degrees of freedom = 1)		

adiposity without morphologic evidence of diabetes mellitus or pyometra.

A 2.0 per cent sample of dogs licensed in Stockholm during the period of the study was used for the statistical analysis. Student's "t" test and χ^2 analyses were performed and Yate's correction applied when indicated. The results were interpreted according to Cramer[2]: * almost statistically significant; ** statistically significant; *** highly statistically significant.

RESULTS

Age Incidence.—Obesity occurred at a slightly earlier age than diabetes mellitus and pyometra, although the difference is statistically significant only between obesity and pyometra (table 1).

TABLE 3—Frequency of Obesity in Different Breeds

Breed	I No. of obese dogs (n = 971)	II No. of dogs of this breed in the general dog population 2% sample (n = 5938)	Comparison between columns I–II χ^2 — value (d.f. = 1)[1]	Significance
Rottweiler	52	91	60.14	***
St. Bernard	14	8	40.74[2]	***
Swedish Hound	25	77	11.27	***
Collie	65	257	10.52	**
Scottish Terrier	64	268	7.83	**
Newfoundland	9	21	5.07[2]	*
Chow Chow	8	19	4.20[2]	*
Mongrel	102	528	2.64	.....
Spitz	69	346	2.44	.....
French Bulldog	5	19	2.03[2]	.....
Norwegian Elkhound	19	85	0.72	.....
Kerry Blue Terrier	3	8	0.68[2]	.....
English Bulldog	4	20	0.56[2]	.....
Doberman Pinscher	7	34	0.29	.....
Barsois	1	1	0.20[2]	.....
Mops	1	1	0.20[2]	.....
Saluki	1	1	0.20[2]	.....
Irish Setter	5	22	0.15[2]	.....
Schnauzer	28	159	0.12	.....
Cocker Spaniel	54	320	0.04	.....
Labrador Retriever	1	2	0.02[2]	.....
Cairn Terrier	3	15	0.00[2]	.....
Great Dane	4	40	0.00[2]	.....
Griffon	2	9	0.00[2]	.....
Toy Pinscher	9	55	0.00	.....
Afghan	1	11	0.02[2]	.....
Boston Terrier	1	12	0.07[2]	.....
Skye Terrier	5	35	0.08	.....
Lakeland Terrier	5	36	0.12	.....
Dachshund	74	484	0.36	.....
Pointer	3	31	0.40[2]	.....
Springer Spaniel	4	35	0.48	.....
Maltese	2	25	0.51[2]	.....
Yorkshire Terrier	1	28	0.59[2]	.....
Sealyham Terrier	3	33	0.96	.....
English Setter	3	35	1.25	.....
Irish Terrier	3	35	1.25	.....
Dachsbrache	2	33	1.38[2]	.....
Welsh Terrier	2	33	1.38[2]	.....
Whippet	1	26	1.62[2]	.....
German Shepherd Dog	75	565	3.16	*
Vorsteh	3	50	4.18[2]	.....
Boxer	57	460	4.24	*
Fox Terrier	100	772	5.56	*
Airedale Terrier	32	340	8.58	**
Poodle	21	265	11.13	***
Pekingese	6	138	11.86	***
Breed not stated	12	.....	.....	.....

[1] d.f. = degrees of freedom. [2] Corrected value (Yate's correction).

Sex Incidence.—Obesity as well as diabetes mellitus predominated in females (table 2).

Breed Incidence.—The breed incidence was compared with the general dog population for: obesity (table 3), pyometra (table 4), and diabetes mellitus (table 5). The summarized results (table 6) demonstrate that roughly the same breeds are over- and under-represented in all three diseases.

Obesity was diagnosed at necropsy in 1,273 of 10,993 dogs (11.6%). Of the 1,273 obese dogs, 971 were obese only, 202 had obesity associated with pyometra, and 100 had diabetes mellitus. Of the nonobese dogs, 67 had diabetes mellitus and 285 had pyometra.

DISCUSSION

The incidence of obesity (11.6%) was greater than that of any other single canine disease in Sweden.

The figures for the frequency of obesity in association with diabetes mellitus and pyometra must be considered as minimal since they represent the nutritional condition of the animals at the time of necropsy only. The condition of many animals was probably much better at the onset of the diabetes or pyometra than at the time of necropsy. Clinical experience and the age analysis of the present material support such a view. The age data revealed that dogs with obesity had the lowest average age, followed by those with diabetes and

TABLE 4—Frequency of Pyometra in Different Breeds

Breed	I No. of dogs with pyometra (n = 487)	II No. of dogs of this breed in the general dog population 2% sample (n = 5938)	Comparison between columns I–II χ^2 — value (d.f. = 1)[1]	Significance
St. Bernard	11	8	61.85[2]	***
Rottweiler	27	91	40.56	***
Chow Chow	8	19	15.75[2]	***
Swedish Hound	16	77	12.48	***
French Bulldog	6	19	7.49[2]	**
Collie	32	275	6.35	*
Pointer	7	31	4.95[2]	*
Great Dane	8	40	4.46[2]	*
Skye Terrier	7	35	3.76[2]	*
Schnauzer	20	159	3.08	.....
Gordon Setter	2	4	2.05[2]	.....
Irish Terrier	6	35	2.00[2]	.....
Airedale Terrier	33	340	0.71	.....
Scottish Terrier	26	268	0.68	.....
Mongrel	47	528	0.55	.....
Griffon	1	9	0.08	.....
Kerry Blue Terrier	1	8	0.05[2]	.....
Cairn Terrier	2	15	0.03[2]	.....
Newfoundland	2	21	0.03[2]	.....
Dachsbrache	3	33	0.02	.....
English Bulldog	2	20	0.02[2]	.....
Doberman Pinscher	3	34	0.01	.....
English Setter	3	35	0.01	.....
German Shepherd Dog	47	565	0.01	.....
Sealyham Terrier	2	33	0.01[2]	.....
Dalmatian	1	5	0.00[2]	.....
Boston Terrier	1	12	0.00	.....
Toy Pinscher	5	55	0.00	.....
Vorsteh	4	50	0.00	.....
Lakeland Terrier	2	36	0.06[2]	.....
Spitz	24	346	0.08	.....
Norwegian Elkhound	6	85	0.12	.....
Whippet	1	26	0.59[2]	.....
Springer Spaniel	1	35	0.86[2]	.....
Cocker Spaniel	17	326	3.26	.....
Pekingese	4	138	5.47[2]	*
Boxer	22	460	6.76	*
Poodle	9	265	7.54	**
Fox Terrier	38	772	11.05	***
Dachshund	19	484	11.25	***
Breed not stated	11	.....	.....	.....

[1] d.f. = degrees of freedom. [2] Corrected value (Yate's correction).

pyometra. The small number of cases of diabetes mellitus precludes drawing any firm conclusions. In the pyometra group, where the onset-to-death time was longer, there were fewer obese dogs than in the diabetes mellitus group where the onset-to-death time was shorter. The indication, therefore, is that both diabetes mellitus and pyometra were preceded by obesity.

The sex and breed distribution indicates that the relationship of diabetes mellitus, obesity, and pyometra is not merely chronological in nature. In both the diabetic and obese groups, there was a significantly greater incidence in females than in males, in spite of the exclusion of the pyometra cases. Wilkinson [9] cited a similar age and sex distribution in his review of diabetes mellitus in dogs. The incidence of diabetes showed a marked peak at 8 years and older. There were 93 females, 46 males, 1 castrated male, 5 castrated females, and 5 sex not stated. Wilkinson [10] also noted that the age incidence of dogs with diabetes mellitus corresponded closely to those with pyometra.

The breed analyses indicate that the same breeds tended to show significant predisposition and resistance to diabetes mellitus, obesity, and pyometra and that the O-hypothesis (breed frequency in general population and diabetes-obesity-pyometra should be the same) is not valid.

This study indicates a definite interrelationship of diabetes mellitus, obesity, and pyometra without providing evidence for a direct cause-effect pattern. Similar conclusions were reached by Larsson [3] in a study of obesity in mice caused by gold thioglucose. He observed an increased incidence of diabetes mellitus in animals obese for a long period. In man, also, diabetes mellitus appeared to be more frequent in obese individuals (Marks [5]).

Although any discussion of etiology must be largely speculative, the available evidence suggests endocrine involvement—primary or secondary—as a factor in what might be called the diabetes mellitus-obesity-pyometra syndrome.

Studies of obesity in man first established the importance of the hypothalamus in the control of food intake. Brobeck [1] has re-

TABLE 5—Frequency of Diabetes Mellitus in Different Breeds

Breed	I No. of dogs with diabetes mellitus (n = 167)	II No. of dogs of this breed in the general dog population 2% sample (n = 5938)	Comparison between columns I–II χ^2 — value (d.f. = 1)[1]	Significance
Rottweiler	19	91	80.56[2]	***
Dachsbrache	6	33	18.98[2]	***
Cocker Spaniel	18	320	9.01	**
Swedish Hound	7	77	8.00[2]	**
Mongrel	23	528	4.71	*
Doberman Pinscher	3	34	2.29	.....
Spitz	13	346	1.12	.....
Irish Setter	2	22	1.10[2]	.....
Whippet	2	26	0.89[2]	.....
Maltese	2	27	0.80[2]	.....
English Setter	2	35	0.25[2]	.....
Dachshund	13	484	0.03	.....
Newfoundland	1	21	0.02[2]	.....
Pointer	1	31	0.02	.....
Cairn Terrier	1	15	0.00[2]	.....
Toy Pinscher	2	55	0.00[2]	.....
West Highland White Terrier	1	15	0.00[2]	.....
Norwegian Elkhound	2	85	0.01[2]	.....
Airedale Terrier	9	340	0.03	.....
Collie	5	257	0.71	.....
Schnauzer	2	159	0.86[2]	.....
Poodle	4	265	1.64	.....
Fox Terrier	16	772	1.68	.....
Scottish Terrier	4	268	1.80	.....
Pekingese	0	138	2.99[2]	.....
German Shepherd Dog	7	565	5.15	*
Boxer	0	460	13.96	***
Breed not stated	2	.....	.....	.....

[1] d.f. = degrees of freedom. [2] Corrected value (Yate's correction).

TABLE 6—Significant Over- and Under-Representation of Diabetes Mellitus, Obesity, and Pyometra in Different Breeds

Breed	Diabetes mellitus	Obesity	Pyometra
OVER-REPRESENTED			
Rottweiler	***	***	***
Swedish Hound	**	***	***
St. Bernard	.....	***	***
Chow Chow	.....	*	***
Collie	.....	**	*
Dachsbrache	***	.....	.....
Cocker Spaniel	**	.....	.....
Scottish Terrier	.....	**	.....
French Bulldog	.....	.....	**
Mongrel	*	.....	.....
Newfoundland	.....	*	.....
Great Dane	.....	.....	*
Pointer	.....	.....	*
Skye Terrier	.....	.....	*
UNDER-REPRESENTED			
German Shepherd Dog	*	.....	.....
Airedale Terrier	.....	**	.....
Dachshund	.....	.....	***
Fox Terrier	.....	*	***
Pekingese	.....	***	*
Poodle	.....	***	**
Boxer	***	*	*

viewed the experimental work which eliminated pituitary lesions as a cause of obesity. The role of endocrine imbalance in diabetes mellitus is well documented, and there is evidence that the cause of pyometra may be ascribed to hormonal imbalance.[8]

On the other hand, the diabetes-obesity-pyometra syndrome might be considered a primary hereditary condition. Mayer [6] studied a family of Shetland Sheepdogs with hereditary obesity appearing as a recessive Mendelian character. Mayer [6] demonstrated a hypersecretion of glucagon and insulin in hereditarily obese, diabetic mice. Bloom and Roberts (cited by Wilkinson [9]) emphasized the importance of a hereditary factor in diabetes mellitus.

In the present study, the appearance of two acromegalic breeds (Rottweiler and St. Bernard) in the group highly predisposed to diabetes mellitus-obesity-pyometra is of interest. Stockard [7] demonstrated that the pituitary of acromegalic dogs contained larger numbers of acidophils than non-acromegalic dogs. These cells are usually considered to be the source of growth hormone, and the latter is often incriminated as the diabetogenic hormone of the anterior lobe of the pituitary. The presence of the nonacromegalic Swedish Hound in the same highly predisposed group and the absence of the acromegalic Great Dane forces one to view this possibility of a pituitary influence with caution. Wilkinson,[9] however, collected 12 dogs with diabetes mellitus and examined their pituitary glands histologically. Three glands were normal, one showed hypertrophy, and eight showed an increase in acidophils.

SUMMARY

In a statistical review of necropsies on 10,993 dogs, 971 were obese, 487 had pyometra, and 167 had diabetes mellitus. Obesity, with an incidence of 11.6 per cent, was the most frequent disease of dogs in this series.

Obesity tended to occur at a slightly earlier age than diabetes mellitus and pyometra. Significantly greater numbers of females than males were affected by obesity and diabetes mellitus. Analysis of breed distribution in relation to the general dog population revealed that certain breeds were significantly predisposed or resistant to diabetes mellitus, obesity, and pyometra. Predisposition or resistance to any of the three diseases was usually associated with predisposition or resistance to all three of the diseases.

The etiologic implications of this diabetes mellitus-obesity-pyometra syndrome are discussed.

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An *in Vitro* Method for the Identification of Strains of Newcastle Disease Virus

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THE ADSORPTION of Newcastle disease virus by suspensions of chicken embryo brain cells can be determined by measuring the hemagglutination activity remaining in the supernatant fluid. The avidity of strains of virus for attachment to brain cells varies and may be used as an identifying characteristic.

MATERIALS AND METHODS

Strains of Newcastle disease virus used in these experiments were from the repository at the University of Wisconsin, Madison. Prior to use they were passed at least twice at the $l.d._{50}$ dilution in 10-day chicken embryos to insure purity of the stock. The stock virus was then prepared by inoculating 0.1 ml. of a 10^{-2} dilution of virus by the allantoic chamber route. Harvest of allantoic fluid was made at death of the embryo, 44 to 120 hours postinoculation. The fertile eggs were supplied by a commercial hatchery whose flocks during this period were free of infectious disease. Incubation prior to inoculation was at 38 to 39 C., humidity was 60 per cent, and the eggs were turned every two hours.

The brain-cell suspensions were obtained from 10-day normal chicken embryos. The brains from 100 embryos, removed by squeezing the head firmly between the fingers, were allowed to drop into sterile Petri dishes. Brains were then placed into 250-ml. screw-capped prescription bottles containing about 150 ml. of physiological saline solution (pH 6.7 to 7.3) and shaken in a vigorous up-and-down movement until the membranes were detached and floated to the surface. The saline wash and membranes were then discarded. This was repeated five to seven times in fresh saline solution until the washings became clear. The brains then appeared completely white, free of blood and membranes.

After the last washing, brain tissues in 20 ml. of saline solution were vigorously macerated by pumping them in and out of a 2-ml. automatic pipette. The suspension was poured into 50-ml. plastic centrifuge tubes and washed three times in 0.85 per cent saline solution after sedimentation at 4 C. in a Sorvall centrifuge* at 5,000 r.p.m. The washing freed the brain suspension of red blood cells. After the last 15-minute centrifugation cycle, the cells were packed at 5,000 r.p.m. for 30 minutes.

The tubes then contained three distinct cell layers. A red layer at the bottom contained red blood cells, a pink layer above this consisted of both red blood cells and microglial cells, and the top layer was white, free of red blood cells, and contained microglial cells and brain cell fragments. Only the white surface layer, collected by carefully pouring or pipetting, was used for adsorption experiments. The brain-cell suspension was then packed by centrifugation at 5,000 r.p.m. for 15 minutes and stored at 4 C. with 1,000 units of penicillin and 1 mg. of streptomycin per milliliter of packed cells. Desired concentrations were prepared by measuring a specific volume of packed cells in a graduated pipette and making the appropriate dilutions in physiological saline solution. Cells were used within one day following preparation.

The avidity of the strains of Newcastle disease virus was measured by a hemagglutinative depletion procedure. The first step was to mix 0.5 ml. of undiluted virus with 2.0 ml. of a 10 per cent suspension of brain cells in physiological saline solution. Immediately after mixing the virus and brain cells, the preparation was pipetted three or four times and then centrifuged at 5,000 r.p.m. for 15 minutes at 4 C. Virus adsorbed on brain cells was removed by sedimentation of the cells. The virus remaining in the supernatant fluid was measured by the Salk pattern method of hemagglutination titration.[3] A control titration of virus, present before adsorption, was prepared from a tube containing 2.0 ml. saline solution and 0.5 ml. of the virus amnioallantoic fluid. The difference between the amount of virus put into the system (the control) and the unadsorbed virus (supernatant fluids after centrifugation) represented the amount of virus adsorbed by the suspension of brain cells.

RESULTS

For comparative purposes, the results are reported as the per cent of hemagglutinating activity adsorbed by brain cell suspension. The calculation procedure is shown (table 1). Two observation periods were employed: (1) following centrifugation of the preparation immediately after mixing and (2) following centrifugation of the preparation 30 minutes after mixing. The data reported are the averages of five observations using strains Eng.-Herts-

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* Sorvall centrifuge, manufactured by Ivan Sorvall, Inc., Norwalk, Conn.

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TABLE 1—Calculation of the Percentage of Hemagglutinating Activity Adsorbed from Differences in Titers of the Original and Adsorbed Virus

Original HA titer	HA titers after adsorption	Per cent of HA adsorbed
160	80	50
160	40	75
160	20	88
160	10	94
160	5	96
160	0	100

1933 (Huntington), Texas-GB-1948, NY-Jones-1948, B1, Mass-MK107-1949; three observations using Calif-RO-1944, Eng-F (ARS), Wis-VLWI-1951, NJ-Roakin-1946 (FRB); and two observations using strains Ont.-Cleroux-1946, Italy-Milano-1945, and Del-Hickman-1946. The results varied ± 10 per cent (table 2).

Adsorption on cells of the central nervous system might be assumed to be a factor in the pathogenic potential of a strain. In work reported elsewhere,[2] we have shown that a culture of low virulence for chickens may be selected from a strain of moderate virulence (Iowa-125-1947) by subjecting the inoculum to adsorption by a suspension of brain cells at each of five successive chicken embryo passages. The derived strain from unadsorbed virus induced an inapparent infection which, nevertheless, protected 5-week-old chickens against a lethal challenge with the GB strain.

The avidity for brain cells of the 12 strains tested can be used to support, in part, the relationship between brain-cell receptors and pathogenicity for chickens. All of the strains of high pathogenicity for chickens (Calif-RO, Ont-Cle., Milano, Herts, and GB) were readily adsorbed. Strains of lesser pathogenicity gave more erratic results. Roakin, Jones, VLWI, and MK107, which seldom cause apparent infection in 100-week-old chickens when inoculated intravenously ($l.d._{50} = 0$) and which normally kill day-old chicks when inoculated intracerebrally (intracerebral pathogenicity index = 0.5 >),[1] were adsorbed to the extent of 87 per cent (Roakin), 62 per cent (Jones), and 75 per cent (VLWI) or not at all (MK107). The two remaining strains, Eng-F and B1, produced inapparent infections in both 10-week-old chickens by the intravenous route and in day-old chicks by the intracerebral route. Yet the F strain is almost completely adsorbed, while B1 only was slightly adsorbed.

These results may be explained on the basis that affinity for the surface substances of brain cells is only one of several characters which cause pathogenic strains to be neurotropic, and that it requires the presence of some other as yet unknown factor in order to confer pathogenicity.

TABLE 2—The Percentage of Hemagglutinins Removed by Adsorption on Brain Cells for 12 Strains and the Relationship of the Adsorption on Brain Cells to Pathogenicity for Chickens

Strain of virus	Per cent of HA adsorbed		Measure of pathogenicity	
	0 min.	30 min.	Chicken $l.d._{50}$	ICP index*
Calif-RO-1944	100	100	8	1.6
Eng-F (ARS)	97	99	0	0
Ont-Cleroux-1946	94	100	4	1.7
Italy-Milano-1945	87	95	8	1.8
NJ-Roakin-1946 (FRB)	87	90	0	1.0
Eng.-Herts-1933 (Huntington)	85	95	8	1.9
Texas-GB-1948	85	90	8	1.9
Wis-VLWI-1951	62	75	0	0.6
Del-Hickman-1946	50	85	0	1.9
NY-Jones-1948	62	0	0	1.3
B1	25	50	0	0
Mass-MK107-1949	0	0	0	1.7

* Intracerebral pathogenicity index.[1]

The receptor substance on brain cells appears to be distinct from the receptor substance on fowl erythrocytes, as the hemagglutinating activity of some strains (MK107) is not removed by an exposure that completely removes the activity of other strains (Calif-RO). Certain pairs of strains which resemble each other in many characteristics can be distinguished by the brain-cell adsorption test; MK107 with an adsorption of 0 and Roakin with an adsorption of 87 are such a pair.

SUMMARY

Suspension of chicken embryo brain cells were found to adsorb all the hemagglutinating activity of some strains of Newcastle

disease virus and to leave the hemagglutinating activity of other strains unaffected. Pathogenicity of strains of Newcastle disease virus for chickens appears to be related in part to the avidity of the virus for brain cells. The procedure described can be used to study the nature of pathogenicity and can also be used as a characteristic in the identification of strains of Newcastle disease virus.

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Sequential Changes in the Titer of Newcastle Disease Virus in Tissues—A Measure of the Defense Mechanism of the Chicken

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A CHICKEN infected with a highly pathogenic strain of Newcastle disease virus usually dies within four to five days. The signs of disease, which include respiratory distress, incoordination, tremors, and paralysis, appear late on the second or early on the third day. Virus is known to spread from the site of entry; it increases in titer in many tissues during this period and usually continues to increase until the time of death. The invasion of selected tissues by the virus and the multiplication of the virus in these tissues can be specifically related to the course of disease by killing individuals at selected intervals following infection and titrating the viral content of the tissues. Using this quantitative procedure, conditions that alter the course of infection can be studied with greater understanding.[1]

MATERIALS AND METHODS

Virus.—Two strains of Newcastle disease virus obtained from the repository maintained at the University of Wisconsin were used in these studies: Texas GB-1948 and Italy-Milano-1945. Both are highly pathogenic strains isolated from birds with field infections. The material employed was pooled amnioallantoic fluid having a chicken embryo l.d.$_{50}$ of $10^{10.5}$ for both GB and Milano and a chicken intravenous l.d.$_{50}$ of $10^{9.4}$ for GB and $10^{10.2}$ for Milano. Inoculum for the experiments was stored at −10 C. in 5-ml. aliquots.

Experimental Chickens.—Crossbred White Leghorn-New Hampshire Red chickens, 7 to 12 weeks old, were obtained as 1-day-old chicks from the Poultry Department of the University of Wisconsin. The source flock was free of Newcastle disease. The chicks, raised in isolation and normal in behavior, were shown to be free of antibodies to Newcastle disease at the time of the experiment.

The chickens were inoculated subcutaneously with a predetermined dose of virus. Every four or six hours, 2 chickens selected at random were removed from the inoculated group, killed, and representative tissues were taken. Virus content of the tissues was determined by chicken embryo inoculation. The inoculated birds that remained were examined for signs of disease. Uninoculated control birds and controls for special treatments were also maintained.

Chicken Embryos.—For preliminary incubation * of chicken embryos the temperature was kept at 39 C. and the humidity at 60 per cent. Fertility at ten days was approximately 70 per cent. Incubation after inoculation was at 37 C.

The weighed tissues were ground with tryptone broth in a TenBroeck grinder and appropriate dilutions of the tissue suspensions were prepared by adding broth. Ten-day embryos were inoculated in the allantoic chamber with 0.1 ml. of the selected suspension of virus-containing tissues. The inoculated eggs were candled daily until death of the embryo or for six days. All embryos dying before 36 hours were discarded as nonspecific. Those remaining alive at six days were discarded as noninfected. The amnioallantoic fluids of all embryos dying within the specified period were tested for bacterial contamination by inoculating nutrient agar slants and thioglycolate broth. Virus content was determined by use of the hemagglutination test.

Hydrocortisone.—The hydrocortisone (Hydrocortone acetate * and Compound F †) dosage was adjusted according to the weight of the bird. Over 50 per cent of 35 chickens (weighing 1.3 to 1.8 kilograms and given hydrocortisone at the rate of 1 mg. per kilogram of body weight per day) lost an average of 130 Gm. each in five days of treatment.

PROCEDURES AND RESULTS

The first experiment sought to answer the question, "how rapidly does the virus spread to various tissues in the body?" Eighty-eight chickens, 10 weeks old, were each inoculated with 0.5 ml. of a 10^{-5} dilution of the GB strain subcutaneously in the left breast. Six chickens were used as inoculated controls and 30 were used to titrate the virus. Starting at the zero hour, 2 chickens of the primary group were killed every four hours for 84 hours. The tissues

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* James Model 252 incubator, manufactured by James Manufacturing Co., Fort Atkinson, Wis.

* Product of Merck Sharp and Dohme, Philadelpha, Pa.

† Product of Merck & Co., Inc., Rahway, N.J.

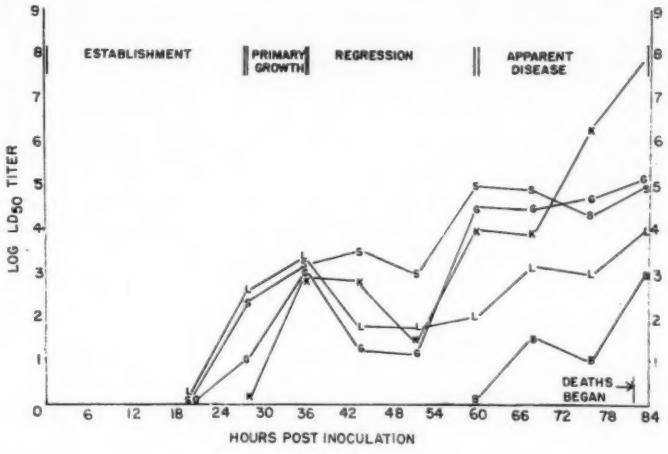

Fig. 1—Titer of the Texas-GB-1948 strain of Newcastle disease virus in tissues of infected chickens. B = brain, G = lung, K = kidney, L = liver, S = spleen.

were frozen until tested. Muscle was taken from the inoculation site. Other tissues taken were the left quadriceps femoris muscle, the left lobe of the liver, spleen, left superior kidney, pancreas at the angle of the small intestine, small intestine approximately 2 inches below the pancreas, esophagus, bursa of Fabricius, inferior lobe of the left lung, trachea, air sac (if obtainable), blood at the site of the break in the neck, cord at the neck level, and cerebrum. All tissues were treated in a similar manner. They were weighed, triturated in nutrient broth to make a 1 to 10 dilution, and antibiotics were added. After one half hour, 0.1-ml. quantities of the preparation were inoculated into the allantoic cavities of 6 chicken embryos.

Virus was detected at the inoculation site immediately after inoculation and at all subsequent time intervals. Virus became detectable in the spleen, lung, and liver about 24 hours after inoculation. With the exception of the cerebrum, cord, and pancreas, the tissues examined contained virus within 48 hours. All tissues became positive within 72 hours.

Still unanswered was the question concerning the titer of the virus in the tissues at intervals after inoculation. Serial dilutions of the tissue suspensions were prepared and their infectivity determined in embryos. The titration results of five tissues—spleen, kidney, liver, lung, and brain—are shown (fig. 1). Virus which became detectable in the viscera after approximately 24 hours increased in titer for the next 12 hours. A depression occurred after the thirty sixth hour and lasted for a little more than 12 hours. The titer then increased in all infected tissues. This increase continued in most instances until the time of death. Virus was not detected in the brain until the titer in the visceral tissues increased following its temporary regression. This was 68 hours after inoculation, and signs of Newcastle disease occurred in birds reserved for observation at this time. There were minor individual variations in signs of the disease in the chickens, and deaths occurred over a 16-hour period beginning 80 hours after inoculation and ending at 96 hours.

It was expected that the titers in the tissues would increase without appreciable interruption from the time of their first appearance until the time of death of the host. The regression of virus titer which occurred in the non-nervous tissues before the virus invaded the brain was striking for several reasons. In most instances, the decrease was simultaneous and marked; tenfold to one-thousandfold less virus being found in tissues sampled as compared with the titer of the previous set of samples. The significance of the depression in titer between postinoculation hours 36 and 48 was next investigated. Did this mean a decrease in virus in the body?

The experiment was repeated with a few modifications. The Milano strain was substituted for the GB strain. Only seven tissues (brain, liver, spleen, blood, kidney, muscle, and lung) were harvested, and the

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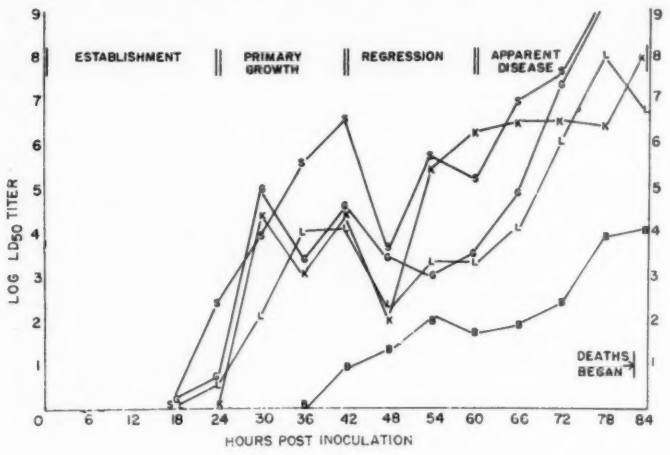
ESTABLISHMENT
PRIMARY GROWTH
REGRESSION
APPARENT DISEASE
DEATHS BEGAN
LOG LD50 TITER
HOURS POST INOCULATION
0 6 12 18 24 30 36 42 48 54 60 66 72 78 84
1 2 3 4 5 6 7 8 9

Fig. 2—Titer of the Italy-Milano-1945 strain of Newcastle disease virus in tissues of infected chickens. B = brain, G = lung, K = kidney, L = liver, S = spleen.

observation interval was increased from four to six hours. The results were similar (fig. 2). After a lag of about 24 hours, virus increased rapidly in the non-nervous tissues, with a temporary regression in titer in most instances at about the 42nd hour and lasting 12 to 18 hours. This was followed by a second increase. Virus was detected in the brain during the period of regression.

It was unlikely that the regression in virus titer was a sampling artifact caused by variation in the resistance of individual chickens to the virus, since it is improbable that in two trials a sequence of pairs would occur among susceptible birds sampled over a period of 24 to 48 hours as the titer increased and among resistant individuals sampled over a period of 40 to 66 hours as the titer remained static or decreased. Comparisons can be made among titers obtained at different postexposure times (14 to 21 hr.) for samples of a given tissue and between trials (2 hr.). The validity of the observations is dependent on the number of samples or trials and not the number of individuals used in a sample. Parker,[3] employing GB, Kan. Manhattan-1948, and NJ-Roakin-1946 strains of Newcastle disease virus, made further trials in which the regression of titer was observed.

DISCUSSION

References to a stage in which growth of virus was arrested in nonimmune hosts were not found in the literature, although such a stage has been observed in bacterial infections. The absence of prior reports is not surprising, since few of the experiments on quantitative pathogenesis have been conducted beyond the first five to ten hours of infection. Most of the experiments covering the later stages of infection have used a longer interval between observations (24 to 48 hours) that would not permit the detection of transitory changes in virus populations.

The arresting or reduction of virus titer could well be an expression of the nonspecific response of the host to a stress induced by events associated with multiplication of virus. Destruction of cells by virus, just as destruction by trauma or chemicals, could release the adaptation syndrome.[2] The fact that the depression in titer occurred in most tissues at approximately the same time indicates that it may be mediated by the entire host and is not the result of a local cell-virus interaction. The adaptation syndrome, triggered by the introduction of a sufficient quantity of foreign virus protein, may result in the removal or inactivation of virus being produced without inhibiting its production. Eventually the mechanism fails from exhaustion.

There would seem to be no reason to suspect the intervention of specific antibody. Although antibody production may be initiated almost immediately on stimulus of the proper cells, antibodies for Newcastle disease virus have not been detected in chickens earlier than four days and are rarely found before seven days. Furthermore, antibody, once it is present in suffi-

cient quantity to affect the spread of virus, should be further augmented in quantity and play an ever-increasing role. The inhibitory mechanism observed is, on the contrary, temporary. Virus multiplication and dissemination is re-established after the recession and usually continues until the host dies.

One method of investigating the mechanism, to determine whether a nonspecific defense raised by the host in response to trauma is the reason for regression of virus titer, is to introduce an anti-inflammatory substance such as cortisone. If the regression is determined at least in part by the Selye syndrome, hydrocortisone should interfere with the inhibitory mechanism. The infections of two groups of chickens, those in one group given GB alone and those in the other given GB virus and hydrocortisone, were followed in the same way as in the two preceding experiments. Two chickens were killed every six hours and the selected tissues titrated in chicken embryos.

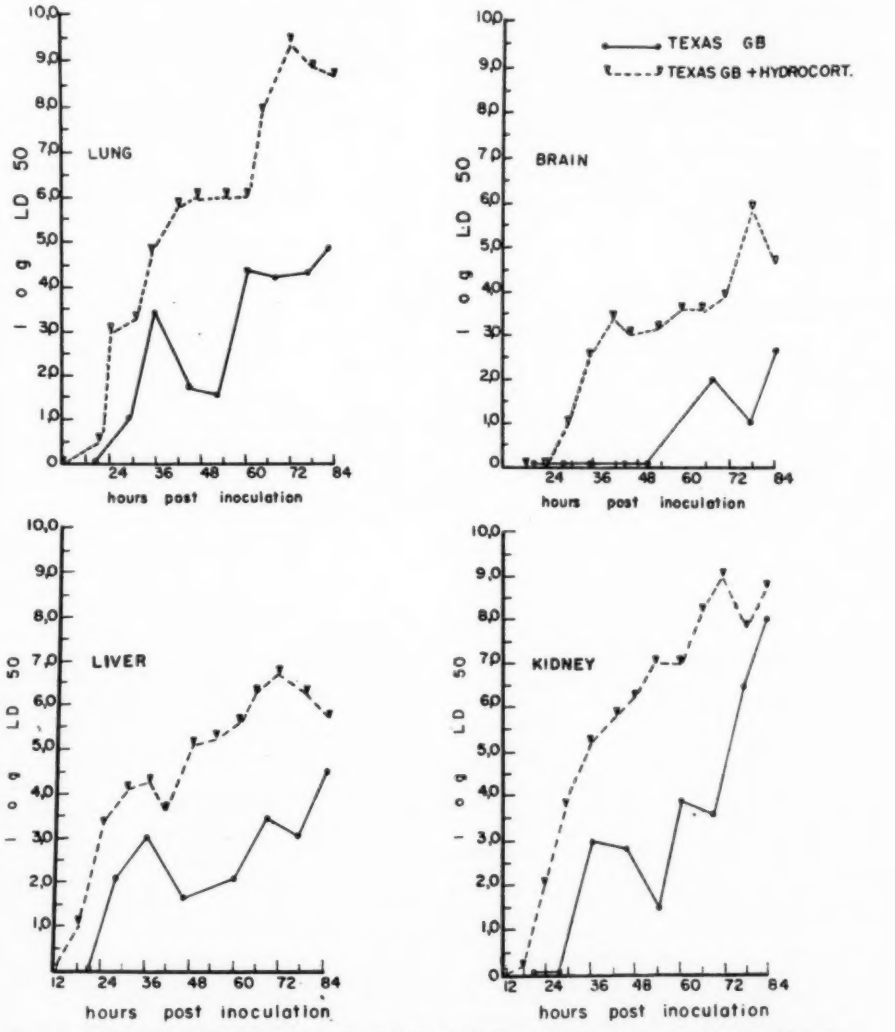

Fig. 3—The effect of hydrocortisone on the titer of Newcastle disease virus in infected chickens.

Hydrocortisone altered the reaction of the chicken to Newcastle disease infection (fig. 3). Signs appeared earlier and death occurred earlier in those in the treated group. The period of regression which was observed in birds in the untreated group was delayed and reduced or was not evident in those in the treated groups.

CONCLUSIONS AND SUMMARY

Newcastle disease virus, when introduced into susceptible fowl, rapidly spread from the site of inoculation and could be detected in almost all tissues from 14 sites in the body within 48 hours and in all the tissues within 72 hours.

Virus titers for eight tissues were obtained at six-hour intervals. The increase in virus titer of the invaded tissues was usually interrupted at about the 36th hour, and this interruption lasted 12 to 24 hours. After the period of interruption, invasion of the central nervous system occurred and signs of disease became evident.

Injection of hydrocortisone usually eliminated the period of interruption and shortened the period between injection and appearance of clinical signs and eventual death.

The temporary interruption of the increase of virus titer in the tissues of infected chickens may be the result of a partially successful adaptation response of the host to the introduction of a foreign protein.

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A Comparison of the Effects of Four Strains of Newcastle Disease Virus on a Strain of Human Epithelial-Like Cells (Maben) and Strain L Mouse Fibroblasts

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NEWCASTLE DISEASE VIRUS (NDV) has been shown to multiply and cause cytopathogenic changes in cell cultures derived from different host species.[1-6, 8, 9, 11-14, 16, 19, 21]

This paper describes the propagation and cytopathogenicity of four different strains of NDV in monolayer cultures of strain Maben [10] human epithelial-like cells, derived from an adenocarcinoma of lung, and Earle's strain L mouse fibroblasts.[7, 18]

MATERIALS AND METHODS

Cells and Medium.—Maben cells* were adapted to growth in a medium consisting of Hanks' balanced salt solution (BSS), 20.0 per cent horse serum (HoS), and 0.1 per cent yeastolate (Difco) with 100 units of penicillin and 100 μg. of streptomycin per ml. Strain L mouse fibroblasts† were propagated in a medium consisting of 90.0 per cent BSS, 10.0 per cent HoS, and 0.1 per cent yeastolate with the same concentration of antibiotics.

The cells were exposed to virus in a maintenance medium designated yeast extract basal medium,[20] which contained 10.0 parts of 1.0 per cent yeastolate in distilled water, 2.5 parts 10.0 per cent glucose in distilled water, and 1 part of 1.13 per cent sodium acetate and 0.64 per cent sodium pyruvate in distilled water. Antibiotics were added in the same concentration.

The cells were propagated in 200-ml. screw-capped bottles. After sufficient growth of the cells had been obtained, they were harvested with 0.2 per cent trypsin (Difco 1:250 trypsin in a calcium- and magnesium-free buffered salt solution), washed, and resuspended in the growth medium in a concentration of approximately 100,000 cells per milliliter. Screw-capped 16 x 125 tubes were then inoculated with 1-ml. amounts each and incubated at 37 C. After sufficient monolayer formation, the growth medium in each tube was replaced with the maintenance medium for addition of the virus.

Virus.—The strains used in this study were Texas-GB-1948, Kan. Manhattan-1948, NJ-Roakin-1946, and B1. The initial inoculums of the strains into cell culture consisted of undiluted allantoic fluid of embryonating chicken eggs, possessing a high hemagglutination (HA) titer and a high 50 per cent embryo lethal dose ($e.l.d._{50}$)[17] titer for all strains except B1.

Propagation of Virus.—Each strain of the virus was inoculated into three tubes of Maben cells. The virus strains were inoculated in 0.1-ml. amounts into each culture tube containing 0.9 ml. of the maintenance medium, and the cultures were incubated at 37 C. The tubes were examined for cytopathogenic effects every 24 hours. After the lapse of 48 hours, the cytopathogenic effects were observed to be maximal. The culture medium in the three tubes was then pooled and the further serial passages were similarly made. The $e.l.d._{50}$ and HA titrations were carried out with the culture harvest, after which it was stored at −20 C. Similar titrations were carried out at different passage levels. Marked cytopathogenic effects were noticed as early as 24 hours and starting from the fouth passage further serial passages were made after making a 10^{-3} dilution of each culture medium. From then on, harvesting of culture medium was done between 72 to 96 hours. Using cover slips in the tubes at various passage levels, the cells were stained by the May-Greenwald method and examined.

The original and last cell culture passage virus of the GB and Manhattan strains were inoculated intratracheally in 0.2-ml. amounts into 2-month-old chickens to determine the pathogenicity and serologic response.

Growth cycle studies were performed using bottle cultures of strain Maben and strain L cells. After the cells reached maximum growth they were washed with BSS and 9.9 ml. of the maintenance medium was introduced. Then 0.1 ml. of an appropriate dilution of the virus was added, mixed, and 0.4 ml. was immediately withdrawn for titration. At 24-hour intervals over the next five-day period, 0.4 ml. of the culture medium was removed and titrations performed.

Using cytopathogenicity as the criterion for determining the end point, the 50 per cent tissue culture infective dose ($t.c.i.d._{50}$) was determined in strain Maben cells. The GB strain of the virus was used in this study, as it was found to produce

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† Obtained through the courtesy of Dr. W. R. Earle, National Institutes of Health, Bethesda, Md.

the most marked cytopathogenic effect. The neutralization indexes for Newcastle disease immune and normal serums were determined as follows: Serial tenfold dilutions of the virus were prepared, starting from a 1:5 dilution of the virus. Then 0.3 ml. of each dilution was mixed with the same volume of undiluted immune serum, undiluted normal serum, or BSS (virus control), mixed, and incubated at 37 C. for 30 minutes. From each dilution in the three series, 0.4 ml. of the mixture was withdrawn and 0.1 ml. of this was introduced into each of four culture tubes containing 0.9 ml. of the maintenance medium. The tubes were read for cytopathogenic effects at frequent intervals; final readings, taken after a lapse of five days, and t.c.i.d.$_{50}$ values were computed.

RESULTS

The initial inoculum of undiluted NDV into Maben cells was followed in 24 hours by pronounced cytopathogenic effects. The GB strain caused a necrosis of the cells and most of them dropped off the glass, leaving behind a cellular debris and a few abnormal cells. The Manhattan strain caused the same effect but to a slightly milder degree. The Roakin strain took two to three days to achieve the same effect. With B1, the cellular damage proceeded more slowly and only about 50 per cent of the cells floated into the medium by the end of five days (fig. 1).

The initial changes consisted of clumping of the cells followed by swelling, cytolysis, and nuclear fragmentation. Starting from the fourth passage, the pronounced effect of the pathogenic strains become milder, and complete necrosis was observed only between 72 to 96 hours, probably due to the 10^{-4} dilution effected with each further passage. With Roakin, destruction of about 75 per cent of the cells occurred at this time, and floating of the cells into the medium was not a characteristic feature. With B1, only partial cellular damage was evident at 72 to 96 hours; cell destruction was more or less complete after the lapse of eight days, with most of the cells still adherent to glass.

The cytopathogenic effects produced by the serially propagated strains were well correlated with e.l.d.$_{50}$ and HA titrations (table 1). Lower e.l.d.$_{50}$ values were obtained for Roakin than for GB and Manhattan; HA titers were lower and sometimes absent. The HA titers of B1 were the lowest and absent in most instances. The presence of the virus was determined here by inoculating eggs and determining the presence of hemagglutinin in the allantoic fluid.

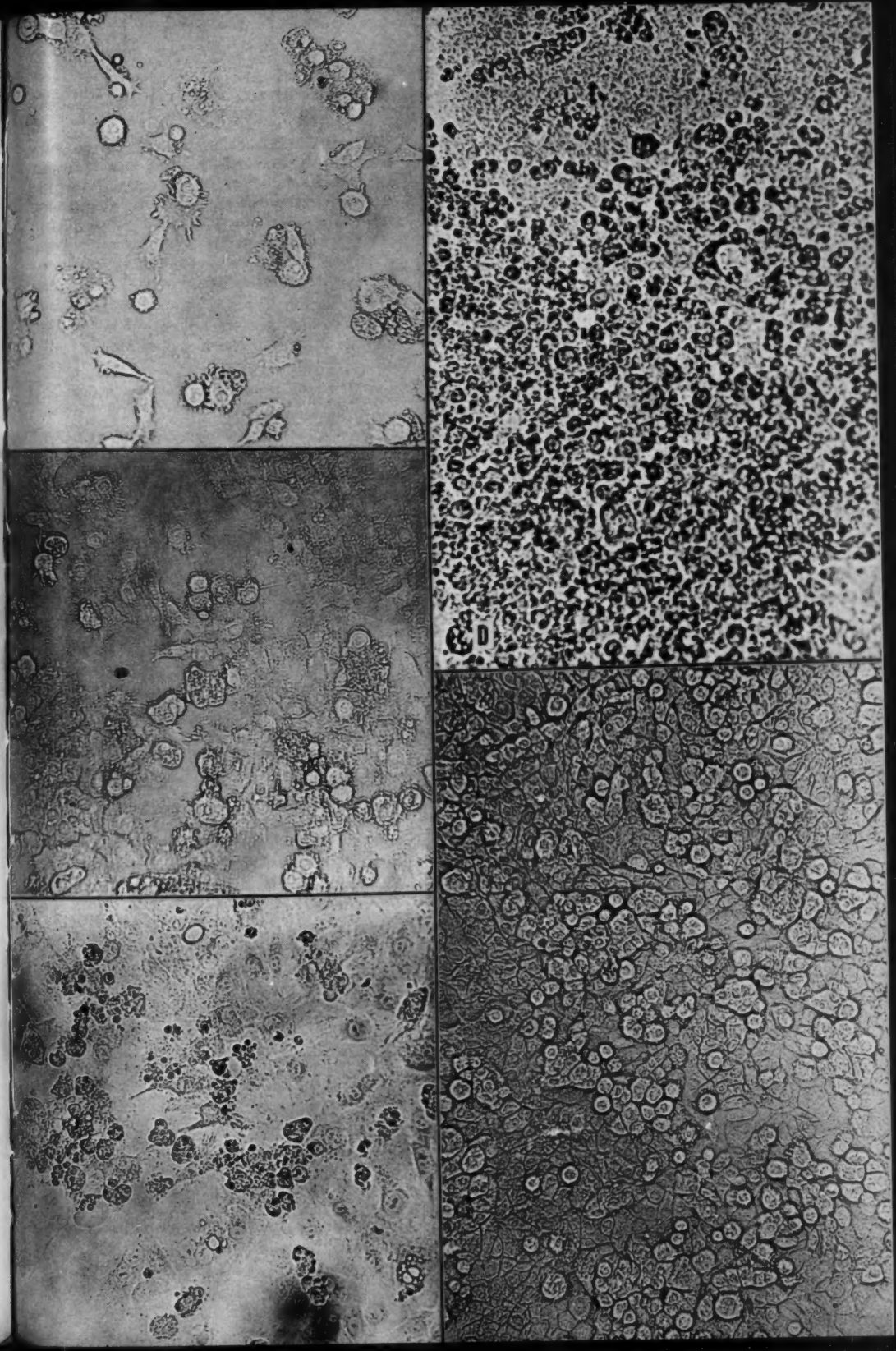
Quite unlike Maben cells, strain L cells did not seem to show any noticeable effect on inoculation with undiluted virus strains. A slight clumping with a few shrunken cells seemed to be evident about six days after inoculation; this remained the same on further incubation. The virus was recovered from third passage.

Growth cycle experiments in Maben cells (table 2) revealed an initial lag period of 24 hours for the GB and Manhattan strains. This was followed by a steady increase in concentration of virus over the rest of the five-day period, with the highest value (over 4½-log increase in titer) for strain Manhattan. An increase in concentration of about 2 logs over the first three days for the Roakin strain and 1 log for the first 24 hours for B1 occurred without a subsequent drop in titer over the rest of the period. With the attainment of sufficient virus concentration, the GB and Manhattan strains gave a positive HA titer on the fourth and fifth day postinoculation.

A similar experiment, using strain L cells, did not result in evidence of viral multiplication over the five-day period. Instead of an anticipated increase in titer, a decrease occurred on the first three days. Whether the virus continued at this same concentration ($10^{3.5}$) or fell lower could not be ascertained, as the e.l.d.$_{50}$ end points fell below the 10^4 to 10^8 range inoculated on days 4 and 5. The HA titers were negative throughout.

Legend for Illustration on Opposite Page

Fig. 1—Cytopathogenic changes observed 24 hours after inoculation of various strains of Newcastle disease virus into Maben cell cultures, shown on the glass walls of test tubes. (A) Normal cells; (B) GB Texas strain—most of the cells have fallen off the glass and the cells remaining on the glass are swollen, shrunken, or appear as cell debris; (C) Kan. Manhattan strain—cells show swelling and fragmentation and many cells have fallen off the glass; (D) NJ—Roakin—the effect is the same as the Manhattan strain but to a milder degree; (E) B1—cells appear shrunken and few have fallen off the glass. x 250.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Strain GB gave a t.c.i.d.$_{50}$ of $10^{9.4}$ in Maben cells. A neutralization index of over 6.5 for Newcastle disease immune serum and only 2.3 for normal serum was obtained.

DISCUSSION

The cytopathogenic effects of NDV in strain Maben cells were well pronounced from the initial passage itself, and no serial passage was required to achieve this effect as reported for strain HeLa cells.[2]

Strain Maben cells would seem to support the growth of the GB strain better than HeLa cells,[2] as judged by the production of cytopathogenic effects at regular intervals after inoculation of virus. Also, there was multiplication of the virus from a diluted state to a concentration sufficient to give HA titers.

The serum neutralization test, using cytopathogenicity for determining end points, can be a sensitive test using Maben cells,

TABLE 1—Multiplication and Cytopathogenicity of Newcastle Disease Virus in Maben Cells

Passage	Cumulative dilution of original virus inoculum	Newcastle disease virus strain										
		GB			Manhattan			Roakin			B1	
		Log e.l.d.$_{50}$	HA	CP*	Log e.l.d.$_{50}$	HA	CP	Log e.l.d.$_{50}$	HA	CP	HA	CP
....	Undiluted original inoculum	10	4,096	.....	9.8	4,096	.....	8.6	4,096	.....	4,096	.....
1	10^{-1}	9.4	512	++++	9.3	512	++++	8.2	512	+++	256	+++
2	10^{-3}	9.5	512	++++	9.2	512	++++	7.2	128	+++	64	++
4	10^{-7}	7.7	128	+++	6.6	64	+++	6.2	32	+++	....	+
8	10^{-23}	6.5	64	+++	7.3	128	+++	5.6	....	++	....	+
12	10^{-39}	6.6	64	+++	6.2	64	+++	6.2	32	++	16	+
16	10^{-55}	6.2	64	+++	6.8	64	+++	5.4	....	++	....	+

* CP = cytopathogenicity. HA = hemagglutination activity.
Plus signs indicate the approximate percentage of cells showing cytopathogenic effects: ++++ = 75 to 100 per cent; +++ = 50 to 75 per cent; ++ = 25 to 50 per cent; + = 10 to 25 per cent.

The differences in the degree of cytopathogenic effects between the strains coincides with their pathogenicity for chickens and is in agreement with similar differences found in divergent strains of NDV in cell culture.[1,2] This characteristic does not, however, seem to be uniform in all types of cells, since Mascoli [13] reported no differences in cytopathogenicity between virulent, vaccinal, and incomplete strains of NDV in chicken embryo fibroblasts.

judged by the high t.c.i.d.$_{50}$ value obtained and the ability of serum to offer complete protection against infection of cells with high concentrations of the virus.

The cell-culture propagated virus appeared complete when harvested prior to maximum cytopathogenic effects.[13,15] (The 16th passage of strains GB and Manhattan produced mild respiratory changes in the birds, and both strains [*beta* procedure] gave rise to hemagglutination inhibition

TABLE 2—Growth Cycle of Newcastle Disease Virus in Maben Cells

Virus strain	Final dilution of virus in medium		Days					
			0	1	2	3	4	5
GB	10^{-5}	Log e.l.d.$_{50}$	2.5	2.4	3.7	4.7	4.8	5.4
		HA	—	—	—	—	—	32
		CP*	—	—	—	+	++	++
Manhattan	10^{-6}	Log e.l.d.$_{50}$	1.5	1.5	3.7	5.4	5.5	6.4
		HA	—	—	—	—	32	32
		CP	—	—	—	+	++	++
Roakin	10^{-5}	Log e.l.d.$_{50}$	3.5	4.4	4.5	5.4	5.5	5.4
		HA	—	—	—	—	—	—
		CP	—	—	—	—	+	++
B1	10^{-5}	Log e.l.d.$_{50}$	2.5	3.5	3.5	3.7	3.5	3.5
		HA	—	—	—	—	—	—
		CP	—	—	—	—	—	+

* CP = cytopathogenicity. HA = hemagglutination activity.
++ = 25 to 50 per cent of the cells show cytopathogenic effects; + = 10 to 25 per cent of the cells show cytopathogenic effects; — = no cytopathogenic effects.

titer of 1/1,024.) Because of the limited number of birds used, it was not possible to determine if there was a change in the pathogenicity of the strains.

Data which are of a preliminary nature do not justify any conclusions on the effect of NDV in strain L cells. However, the cells did not show cytopathogenic changes on initial passages of the virus and did not support the multiplication of the virus on initial contact.

SUMMARY

1) Newcastle disease virus (NDV) caused a marked cytopathogenic effect in strain Maben human epithelial-like cells derived from an adenocarcinoma of lung.

2) There were marked differences in the cytopathogenicity and virus titers attained among the four strains of NDV studied. Strains Texas-GB-1948 and Kan. Manhattan-1948 were the most pathogenic and gave high embryo lethal dose and hemagglutination titers on continuous propagation; strain NJ-Roakin-1946 gave lower titers and B1 the least.

3) The NDV strains were propagated successfully through 16 serial passages in strain Maben cells, representing a final dilution of 10^{55} of the initial inoculum of virus.

4) Serum neutralization tests indicated that cytopathogenicity of virus could be used as a means of estimating relative concentration of antibodies.

5) Preliminary data indicated absence of cytopathogenicity and multiplication of NDV on initial contact with strain L cells.

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The Effect of Green Feed and Ascorbic Acid upon Single Experimental Infections of *Trichostrongylus axei* (Cobbold, 1879) in the Laboratory Rabbit

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THE LITERATURE on the effect of nutrition upon host-parasite relationships has been reviewed by Chandler,[3] Hunter,[11] and von Brand.[1] Recently Vegors *et al.*[19,20] and Riedel and Arnold [14] reported on the effect of diet on nematode parasitism in beef cattle. Gibson [7,8,9] investigated the effects of different planes of nutrition in sheep given multiple infecting doses of *Trichostrongylus axei* (Cobbold, 1879). In general, it has been reported that animals on a low plane of nutrition acquired greater numbers of parasites and were more severely affected than animals on a higher plane of nutrition.

Drudge *et al.*[6] demonstrated that rabbits could be used to study the small stomach worm, *T. axei*. Crampton *et al.*[5] found that rabbits could be used as pilot animals for steers in studying the digestibility of certain constituents of pasture forage.

Therefore, it was decided to use rabbits infected with *T. axei* in preliminary studies of the interrelationship of nutrition and infection with gastrointestinal nematodes of ruminants. The host-parasite system in *T. axei*-infected rabbits was studied in relation to the influence of different rations; *i.e.*, green feed plus a standard pelleted ration, ascorbic acid plus the standard ration, and the standard ration alone. Part of the data reported in this paper has been reported in abstract form.[15]

MATERIALS AND METHODS

Helminth-free grade California white rabbits were raised at this laboratory in cages with wire floors. The rabbits were weaned at 6 weeks of age. When rabbits were allotted to groups, sexes and litters were divided equally among the groups. Each animal was weighed twice a week during the experiment.

Those which were fed only green feed (kudzu, Johnson grass, and tall fescue) were accustomed to it gradually over a period of a week until they were maintained solely on that feed. They were then maintained on the forage for one week prior to infection to obviate any differences that might arise from the change in composition of the ration.

Rabbits which were fed a supplement of alfalfa were given the fresh green feed along with their regular ration for a week prior to infection. Twice each day green feed was cut and fed. At each feeding an excess of forage was given and the remaining old feed removed. The control ration was a standard commercial pelleted rabbit feed (16% protein). Unless otherwise noted, salt and water were available to all animals ad libitum.

The *T. axei* used in this investigation was of bovine origin and was maintained at this laboratory as a pure infection in calves. Eggs were cultured by the method described by Cauthen.[2] The number of third-stage larvae given was determined by a standard dilution counting procedure, and larvae were then administered *per os* by means of a blowpipe. Unless otherwise stated, 6,000 infective larvae were given to each rabbit inoculated. Preliminary experiments had shown that this number of larvae slightly retarded the weight gain in rabbits of this breed, at the ages used.

At necropsy, stomachs were examined for gross pathologic changes. Lesions in each stomach were charted diagrammatically for later reference and comparison. The stomachs were washed in a solution of Tween 80 in warm water to remove the worms. The stomachs were then immediately digested in pepsin-HCl as described by Herlich [10] or were frozen before digestion as described by Ciordia *et al.*[4] Aliquots of both the stomach washings and the stomach digest were taken and examined.

Statistical evaluation was made using Student's "t" test. When percentages were compared, they were first converted to their arc sine equivalents.

PROCEDURE AND RESULTS

EXPERIMENT 1

Kudzu Versus Johnson Grass Versus Standard Ration.—Sixteen 7-week-old rabbits, representing two litters, were allotted to six groups; two groups of 3 rabbits each were fed kudzu, two groups of 3 rabbits each were fed Johnson grass, and two groups of 2 rabbits each were fed the standard ration. Rabbits in the test group (principals) on each ration were inoculated with

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T. axei; those in the other group were held as controls.

Rabbits in both groups on the standard ration gained weight in a normal manner; however, during the experiment the uninfected animals gained an average of 2.5 Gm. per day more than the infected rabbits. Those in groups fed kudzu gained weight slowly, and again the uninfected rabbits gained an average of 2.5 Gm. per day more than the infected animals. Those in groups fed Johnson grass started losing weight as soon as they were on the grass alone.

In the test group on Johnson grass, 1 rabbit died on postinoculation day 14 from obvious malnutrition; consequently, parasite data from this animal are not included in this study. The experiment was concluded when 2 control animals fed Johnson grass died on day 20. At this time, the animals on Johnson grass were below their weaning weight. Prior to the death of the first animal, the weights of the two groups paralleled each other, but by the end of the experiment the uninfected rabbits had lost an average of 3.3 Gm. per day more than did the infected rabbits fed Johnson grass. The data are summarized (table 1).

are not included in table 1, but the numbers of worms recovered from them were comparable to those from the principals killed at the termination of the experiment. One control rabbit fed Johnson grass died on day 16. The experiment was terminated on day 21, as the remaining animals on Johnson grass were below weaning weight and were in extremely poor condition. The data are summarized (table 1).

There was no significant difference in the total numbers of worms recovered from the principals on the different rations in either experiment; however, animals fed green feed had a significantly higher percentage of immature worms than those fed the standard ration.

EXPERIMENT 2

Tall Fescue Versus Standard Ration.— Eleven 7-week-old rabbits from one litter were allotted to four groups, with 3 animals each in the test and control groups on fescue and the test group on a standard ration. Two rabbits were used for the control group on the standard ration. One week after the fescue-fed rabbits had been on a straight grass ration, each principal was inoculated with *T. axei.* Those on fescue continued to lose weight, and by postinoculation day 17, 1 principal and 1 control were moribund and hence were killed. One principal on the standard ration was killed at this time for comparison (table 1). The experiment was terminated at 27 days, as the 4 remaining rabbits on fescue were in extremely poor condition. During the experimental period, the uninfected animals on the standard ration gained an average of 11.1 Gm. per day more than did

TABLE 1—A Comparison of Infections with Trichostrongylus axei in Rabbits on Different Rations (Kudzu Versus Johnson Grass Versus Standard Ration, and Fescue Versus Standard Ration)

		At time of infection		Necropsy			
Ration	No. of rabbits	Age (weeks)	Av. weight (Gm.)	Days after infection	Av. weight (Gm.)	Av. No. *T. axei* recovered	Immature worms (%)
Kudzu	3	9	1,151	20	1,262	1,045	54
Johnson grass	2	9	960	20	809	890	58
Standard	2	9	1,217	20	1,708	875	38
Kudzu	4	9	1,261	21	1,511	265	89
Johnson grass	2	9	1,175	21	906	415	95
Standard	4	9	1,305	21	1,887	360	48
Fescue	1	9	1,067	17	735	1,620	100
Standard	1	9	1,525	17	1,895	1,680	100
Fescue	2	9	1,094	27	914	2,070	17
Standard	2	9	1,322	27	2,046	890	43
Fescue	5	9–15	2,355	20	2,170	955	52
Standard	5	9–15	2,962	20	3,279	830	68

A replication of experiment 1 was conducted with 21 rabbits, representing three litters. The rabbits were allotted to six groups, with 3 animals in each of the control groups and 4 animals in each of the test groups. The weight data resembled closely those of the first experiment, except that there was no difference in weight loss between the principals and controls fed Johnson grass. Two principals fed Johnson grass died on postinoculation days 12 and 13, respectively. Their parasite data

infected animals on the same ration. The infected rabbits fed fescue lost 3.0 Gm. per day more than did their controls; however, until the 2 animals were removed from the experiment on day 17, the two groups on fescue lost weight at an equal rate. The data are summarized (table 1).

A replication was conducted with 10 rabbits from three litters. The rabbits ranged in age from 7 weeks to 3½ months.

A replication of this experiment was conducted with 8 more 7-month-old animals. One week after infection, 2 females from each group were bred. Thirty days after infection, all animals were killed and examined. The pooled data from these two experiments are summarized (table 2).

Fresh alfalfa apparently inhibited the development of *T. axei* in the nonpregnant rabbits. The difference between the mean

TABLE 2—A Comparison of Infections with Trichostrongylus axei in Pregnant and Nonpregnant Rabbits on Different Rations (Standard Ration Plus Alfalfa Versus Standard Ration)

Group and ration	No. of rabbits	At time of infection		Necropsy			
		Age (months)	Av. weight (Gm.)	Days after infection	Av. weight (Gm.)	Av. No. *T. axei* recovered	Immature worms (%)
PREGNANT							
Standard plus alfalfa	3	7	3,236	30	3,294	1,023	10
Standard	3	7	3,784	30	3,813	1,577	21
NONPREGNANT							
Standard plus alfalfa	5	7	3,498	30	3,605	970	54
Standard	5	7	3,057	30	3,170	1,026	15

No uninfected controls were used. This experiment was terminated 20 days after infection, when the first animal fed fescue died. There was no apparent difference in the total numbers of nematodes recovered, nor was there a significant difference in the percentage of immature worms between the two groups (table 1).

EXPERIMENT 3

Green Alfalfa Plus Standard Ration Versus Standard Ration.—As the standard ration contained alfalfa meal, it was decided to supplement the ration of some animals with fresh alfalfa to determine if this would cause an inhibition in development of the parasite as had been observed in the experiments with kudzu and Johnson grass. Eight rabbits, 7 months old, were allotted to two groups; one group was maintained on the standard ration alone, while the other group was given the standard ration plus fresh alfalfa ad libitum twice daily. One week after the latter group was placed on alfalfa, each rabbit in the experiment was given 10,000 infective larvae of *T. axei*. One week later, 1 female from each group plus a male escaped from their cages during the night, resulting in a female in each group becoming pregnant. All animals were killed and examined 30 days after inoculation.

percentages of immature worms recovered from nonpregnant rabbits fed alfalfa and from those fed only the standard ration was significant at the 1 per cent level. There was no significant difference between the percentages of immature worms recovered from pregnant rabbits fed alfalfa and either the pregnant or nonpregnant rabbits fed only the standard ration, nor was there any significant difference between the latter two groups. However, the difference between the percentages of immature nematodes recovered from nonpregnant rabbits fed alfalfa and the pregnant ones fed alfalfa was significant at the 1 per cent level.

There was no statistical difference between the total numbers of nematodes recovered from the various groups.

EXPERIMENT 4

Ascorbic Acid Plus Standard Ration Versus Standard Ration.—The most obvious difference between the standard ration alone and this ration plus green feed was the absence of ascorbic acid in the former, so the substitution of this vitamin in place of green alfalfa was tested. Nine litter mates, 8 weeks old, were allotted to two groups. All were given the standard ration and salt ad libitum and distilled water adjusted to the approximate amount which each rabbit would drink daily. Five rab-

bits in one group were given 10 mg. of ascorbic acid in their drinking water daily, and four days later all 9 animals were inoculated with the parasite. When killed 30 days later, there was no significant difference in the total numbers of nematodes recovered from the two groups; however, almost twice as many immature *T. axei* were recovered from the animals given ascorbic acid (table 3).

A second experiment was conducted with 8 rabbits, 4 months old. Four were each given daily oral doses of 10 mg. of ascorbic acid dissolved in 0.5 ml. of distilled water. The other 4 were maintained as controls. Salt, water, and the standard ration were available to both groups ad libitum. Four days after beginning the administration of vitamin C, all 8 animals were given 10,000 infective larvae of *T. axei;* 30 days later, all 8 were killed and examined. Again, there was no significant difference in the total numbers of nematodes recovered and slightly more than twice as many immature *T. axei* were recovered from the animals given ascorbic acid (table 3).

TABLE 3—A Comparison of Infections with Trichostrongylus axei in Rabbits on Different Rations (Standard Ration Plus Ascorbic Acid Versus Standard Ration)

	At time of infection			Necropsy			
Ration	No. of rabbits	Age (months)	Av. weight (Gm.)	Days after infection	Av. weight (Gm.)	Av. No. *T. axei* recovered	Immature worms (%)
Standard plus ascorbic acid	5	2	1,854	30	2,266	1,690	15
Standard	4	2	1,939	30	2,278	2,080	8
Standard plus ascorbic acid	4	4	2,902	30	3,123	650	13
Standard	4	4	2,766	30	3,010	768	6

When the data from these two experiments were pooled, the difference between the percentage of immature worms recovered from the animals given ascorbic acid and the controls was significant at the 1 per cent level.

There were no differences in weight gains between animals given ascorbic acid and the controls in either experiment.

Observations on Pathologic Changes.—Lesions found in the stomach were typical of those previously described as caused by *T. axei* in the weaned rabbit.[16] Extensive ringworm-like lesions were found in the cardiac region of the stomach, primarily near the entrance of the esophagus, and small nodules were found in the pyloric end.

No differences attributable to the different rations could be found in the pattern or severity of the lesions in the stomachs, for there were greater variations among rabbits within the same dietary group than there were between groups.

DISCUSSION

It is generally considered that a deficiency of protein in the diet of the host is usually beneficial to the establishment of parasitic infections.[1,3,7-9,11] As mentioned earlier, the control ration contained 16 per cent digestible protein. The green feeds had a much lower percentage: kudzu, 4.2; fescue, 1.8; and Johnson grass, 1.1 per cent digestible protein.[13] However, when single infections of *T. axei* were given to rabbits on Johnson grass and fescue, these poor diets had no effect on the total numbers of nematodes recovered at necropsy. Also, the pathologic changes in the stomach were no greater in those animals which were starving to death on a poor ration than in their litter-mate controls which were maintained in good nutritional condition on the standard laboratory rabbit ration. The weight gain data obtained from rabbits on the control ration were closely comparable to data obtained by Gibson[9] from lambs infected with *T. axei* and maintained on hay plus concentrates. However, on the basis of the data Gibson obtained from lambs fed only hay, one would have expected a more rapid weight loss in our infected rabbits fed Johnson grass and fescue than was shown in control rabbits on the same ration. It is probable, in this instance, that the rapid weight loss from a ration of these two grasses overshadowed any debilitating effect that the worms may have had. Therefore, under the conditions of this experiment, it would appear that the percentage of protein in the ration of the host had no effect on the establishment of infection or on the subsequent damage caused by the parasite in this host-parasite system, but that the action of the parasite in this case

was independent of the nutritional state of the host.

This observation is not in accord with data obtained by Gibson.[7,8] He found that sheep experimentally infected with *T. axei* and fed only hay had more worms at necropsy than did comparably infected sheep fed hay plus concentrates. This difference was attributed primarily to the level of nutrition in the two rations. There is a possibility, however, that the difference in infection between Gibson's two experiments was due rather to the difference in the breeds of sheep, since crossbred Down lambs were fed hay alone,[7] whereas Dorset Horn lambs were fed hay plus concentrates.[8] It has been reported by Stewart *et al.*[18] that different breeds of sheep exhibit varying degrees of resistance to *Ostertagia circumcincta*. This might also be true for *T. axei* and other nematodes. Another possible explanation for the difference between Gibson's work with sheep and the present investigation, aside from different hosts being used, is that Gibson gave infective larvae to lambs daily over a 25-day period, while the rabbits reported herein were given a single infective dose. It is possible that animals on a higher protein ration would exhibit a greater resistance to subsequent reinfections with *T. axei* than would animals on a bare maintenance ration.

The data obtained with ascorbic acid are of particular interest, since this vitamin is not considered to be required in the diet of rabbits.[17] All nonpregnant rabbits given this vitamin, with the exception of those fed fescue, had a greater percentage of immature worms than their controls, although the total numbers of worms recovered were comparable. This indicates that ascorbic acid inhibits the development of *T. axei* in the rabbit. It would appear that whatever effect ascorbic acid has, it is blocked by a ration of fescue and by the added strain of pregnancy. This can be regarded as indirect evidence that the results obtained by feeding the vitamin were a manifestation of increased resistance of the host to the helminth rather than a direct action of ascorbic acid on the parasite. Therefore, it would appear from the data obtained that a higher level of vitamin C than can be synthesized by a parasitized rabbit is needed to augment the natural resistance of the rabbit to *T. axei*.

It is possible that the ergot alkaloids present in tall fescue [12] caused a general lowering of the resistance of the host which masked the effect of ascorbic acid. Further, if multiple infections more closely resembling the natural acquisition of parasites had been used, perhaps the rabbits would have demonstrated the same effect as described by Vegors *et al.*,[20] wherein steers pastured on fescue harbored more nematodes at necropsy than steerms maintained on a higher nutritional level.

SUMMARY

When grade California rabbits maintained on kudzu, Johnson grass, fescue, or on a standard pelleted rabbit ration supplemented with alfalfa or ascorbic acid were given a single infective dose of *Trichostrongylus axei*, no more worms were recovered from them at necropsy 17 to 30 days after infection than from control rabbits maintained on the standard ration alone.

Some of the rabbits maintained on fescue or Johnson grass died of malnutrition during the course of the experiment, whether they were infected or not. These poor diets did not enhance the effects of the nematode infection as reflected by weight or gross pathologic changes.

Nonpregnant rabbits fed rations of kudzu, Johnson grass, or supplemented with alfalfa or ascorbic acid had significantly more immature worms than those fed the standard ration. Fescue did not inhibit development of *T. axei*.

Pregnant females fed the standard ration supplemented with fresh alfalfa had significantly fewer immature worms than nonpregnant animals on the same ration. There was no significant difference in the percentages of immature worms in pregnant rabbits fed the standard ration alone or the standard ration supplemented with fresh alfalfa, or in nonpregnant animals fed the standard ration alone.

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Growth of Foot-and-Mouth Disease Virus in Bovine Kidney Cell Suspensions

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THE FEASIBILITY of the use of minced kidney tissue suspended in a simple medium for propagation of vaccinia virus was reported by Maitland and Maitland [6] in 1928. In 1930, Hecke [5] demonstrated that foot-and-mouth disease virus (FMDV) could be propagated in tissue explants. Since then, Frenkel [3,4] and other workers have employed the Maitland method in propagation of FMDV for vaccine production. More recently, the introduction of trypsin as a dispersing agent has made it possible to prepare monolayer cultures for the propagation and assay of virus.[2]

The use of trypsin-dispersed bovine kidney cells in suspension for the production of large quantities of FMDV required for vaccine studies and for physical and chemical characterization of the virus is being explored in this laboratory. This is a report on the growth of FMDV in suspensions of minced tissue, trypsin-dispersed bovine kidney cells, and trypsin-dispersed bovine kidney cells from primary monolayer cultures.

MATERIALS AND METHODS

Virus.—Foot-and mouth disease virus, type A, strain 119, serially passaged 60 or more times in outgrowths of bovine kidney cells, was used in these studies. When fluids from infected cultures were adjusted to a pH of 7.5 and stored in ampules at −40 C., there was no detectable loss in virus infectivity following many weeks' storage. Before use, virus was thawed and clarified at 1,500 r.p.m. to remove cellular debris.

Cell Preparation.—Calves and yearling steers served as sources of kidney tissue. The cortex of the kidney was minced into small fragments (2–4 mm. diameter) and rinsed in chilled Hanks' balanced salt solution. Minced tissue was treated with trypsin to disperse the cells, which were rinsed in chilled phosphate-buffered saline solution, packed by centrifugation at 600 r.p.m. for two minutes, and resuspended in culture medium. Cell numbers were estimated from nuclei counts of cells treated with 0.1 M citric acid and crystal violet.[7]

Culture Preparations.—The trypsin-dispersed kidney cells were used directly for preparation of surviving cell suspensions and for production of monolayer cultures in Roux flasks and 4-oz. prescription bottles. Unless otherwise stated, the medium in suspended cultures consisted of Hanks' balanced salt solution containing 10 per cent bovine serum, Penicillin G (100 units per ml.), and dihydrostreptomycin sulfate (100 μg. per ml.). The medium for Roux flask and 4-oz. prescription bottle cultures was the same, except for the addition of lactalbumin hydrolysate at 0.5 per cent concentration.

Cells that were first grown to confluent monolayers (primary cultures) in Roux flasks were also used for preparation of suspensions. In these cases, cells were loosened from the glass by treatment with trypsin under gentle agitation. They were rinsed and resuspended in culture medium to the desired concentrations.

In most instances, cell suspensions were dispensed in 39-ml. amounts into 250-ml. Erlenmeyer flasks. One milliliter of virus suspension was then added and the flasks were either sealed with rubber stoppers or gassed above the fluid with an air-CO_2 mixture. Suspension cultures were incubated at 37 C. under gentle agitation on a shaker, and samples were removed periodically for virus assay. The reaction of each culture was maintained between 7.1 and 7.4 by the addition of dilute sodium hydroxide or by adjustment of the air-CO_2 mixture.

Assay Method.—The plaque method described by Bachrach *et al.*[1] was used to assay the virus samples. Test material was diluted decimally in culture medium to the desired dilutions. Duplicate 0.1-ml. aliquots from each of the dilutions were transferred to bovine kidney cultures in 4-oz. prescription bottles. After 90 minutes of incubation at 37 C., agar overlay was added, and the cultures were incubated at 37 C. in an inverted position for 72 hours. Titers were recorded as numbers of plaque-forming units (p.f.u.) per milliliter.

EXPERIMENTS AND RESULTS

A comparative study was made of the rate of growth and yield of FMDV in suspensions of minced tissue and in suspensions of trypsin-dispersed cells. Cultures contained about 1 Gm. of tissue either as minced tissue or as dispersed cells and were aerated with an air-CO_2 mixture. The culture medium contained 1 per cent bovine serum. The results (fig. 1) showed that the

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more rapid growth of virus, as well as the higher yield, occurred in the trypsin-dispersed cell suspension. The peak titer in the dispersed cell suspension was reached after 25 hours' incubation, while the minced tissue suspension was incubated 50 hours before peak titer was attained. The maximum yield in the dispersed cell suspension of $10^{7.1}$ p.f.u. per milliliter was about five times greater than in the minced tissue suspension.

The effect of bovine serum on the growth of virus in suspensions of trypsin-dispersed cells was studied in another experiment. Each suspension contained approximately 4.3 million cells per milliliter and was inoculated with virus at a ratio of 0.00056 p.f.u. per cell. The suspensions were maintained at a pH of 7.1 to 7.4 by aeration with an air-CO_2 mixture. The results of this experiment (fig. 2) illustrate the enhancing effect of bovine serum on virus production. In suspensions with serum concentrations of 0, 1, 2, 5, and 10 per cent, the peak titers were $10^{6.2}$, $10^{6.6}$, $10^{6.7}$, $10^{7.1}$, and $10^{7.2}$ p.f.u. per milliliter, respectively. Thus, yields were progressively higher in the presence of greater amounts of serum.

The virus was also grown in a suspension culture with a volume of 10 liters. The cell suspension was contained in a 5-gal. glass carboy and was agitated with a Teflon-covered magnetic bar suspended from a stainless steel wire and swivel assembly. The suspension contained 4.2 million cells per milliliter and was inoculated at a ratio of 0.00033 p.f.u. per cell. Aeration and pH control were again maintained with an air-CO_2 mixture. Results (fig. 3) show that the virus yield, as in the previous small suspensions of dispersed cells, reached a maximum value within 24 hours. The peak titer was $10^{7.2}$ p.f.u. per milliliter.

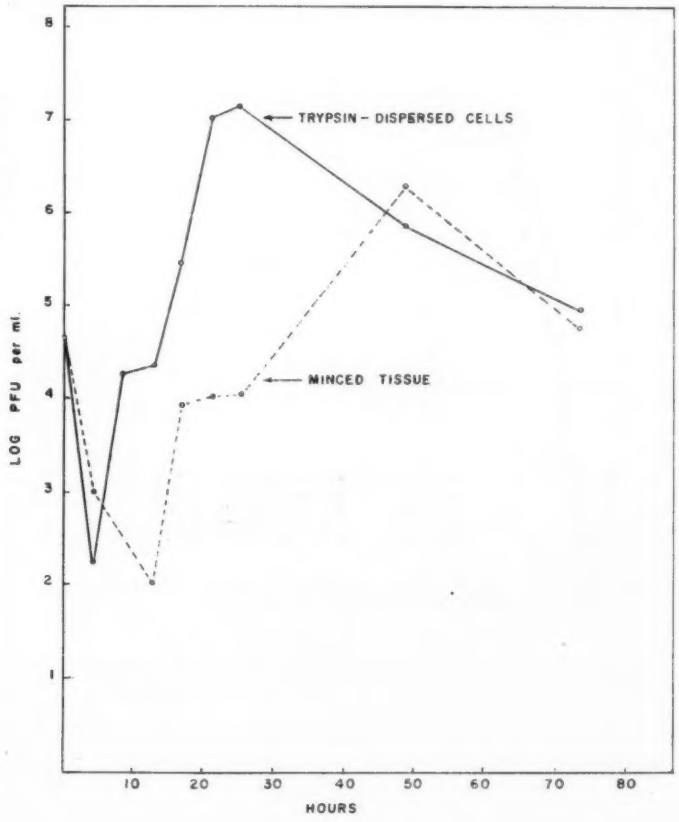

Fig. 1—Growth of foot-and-mouth disease virus A119 in minced bovine kidney tissue and trypsin-dispersed bovine kidney cells in suspension cultures.

In the experiments carried out in cell suspensions derived from primary cultures, the culture flasks were sealed with rubber stoppers and the pH maintained between 7.1 and 7.4 by addition of dilute sodium hydroxide. Peak titers as high as 10^8 p.f.u. per milliliter were reached. Curves (fig. 4) show that in cultures containing the most cells, the maximum yield of virus per cell (19 and 24 p.f.u.) occurred at the 28th hour, while the maximum in the culture containing the least number of cells (60 p.f.u. per cell) was reached at 40 hours.

DISCUSSION

Trysin-dispersed cells were superior to minced tissue for the production of virus. The more rapid growth and higher yield of virus in trypsin-dispersed cells might be attributed to the cells being more accessible to the virus than they are before dispersal. Cells near the center of a fragment of minced tissue are shielded from infection by adjacent cells. Injured or disrupted cells at the surface of a fragment of minced tissue may block the adsorption of virus by the underlying viable cells. The action of trypsin not only separates the viable cells but removes injured cells, thus allowing for better adsorption by cells that support the growth of the virus.

Growth of virus in trypsin-dispersed cells was enhanced by bovine serum. The lowest yield of virus occurred in cultures to which serum had not been added. Progressively higher yields of virus were obtained as the serum concentrations were increased. The highest yield occurred in cultures containing the most serum (10 %).

Large volumes of high-titered virus can be grown either in suspensions of trypsin-dispersed cells or in monolayers prepared

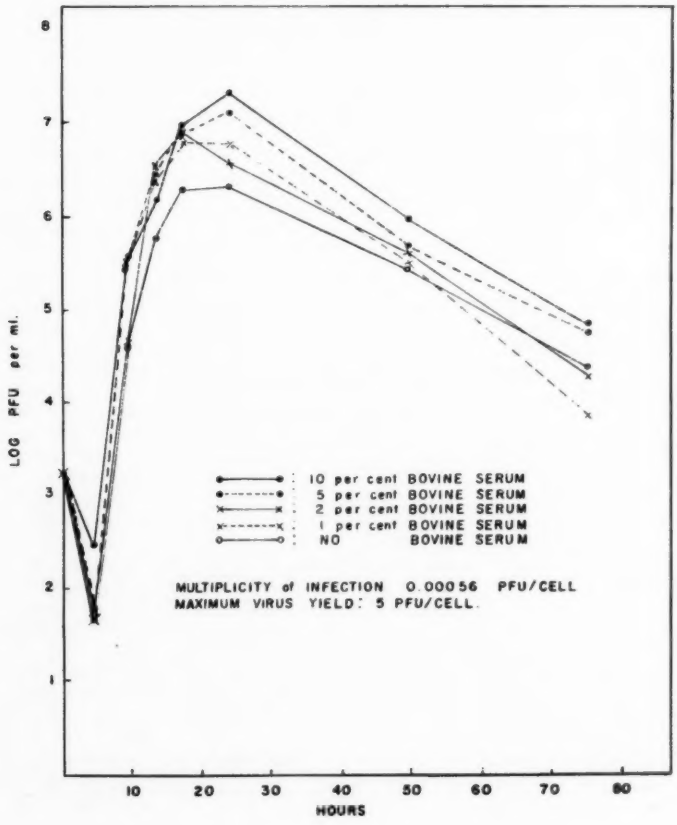

Fig. 2—Growth of foot-and-mouth disease virus A119 in trypsin-dispersed bovine kidney cell suspensions.

from trypsin-dispersed cells. The suspended cell method, however, has several advantages over the monolayer method for the production of virus. Ten liters of high-titered virus were obtained from a suspension of trypsin-dispersed cells 24 hours after preparation of the suspension. If monolayer cultures had been prepared for the propagation of the virus, a week or more additional time would have been required before the virus could have been harvested. The greater initial requirements in glassware and incubator space for production of virus by the monolayer method are multiplied greatly by the increased time this equipment and space are engaged. The use of dispersed cells in suspension for virus propagation is also more economical in culture medium and labor requirements. Finally, many of the trypsin-dispersed cells do not attach to the glass in the monolayer method and are discarded before virus is added. In this laboratory, 80 to 90 per cent of the dispersed cells seeded into culture bottles for growth of monolayers are discarded at the first medium change. However, the suspended cell method compares unfavorably with the monolayer method in yield of infectious units per cell. Maximum yields of between 100 and 400 p.f.u. per cell have been obtained from monolayer cultures, as compared with a maximum yield of 5 p.f.u. per cell from suspended cell cultures.

When suspensions of cells were used for the production of FMDV, highest yields were obtained when suspensions were prepared with cells first grown in monolayers and then treated with trypsin to effect their removal from the glass and dispersal. The average yield of 60 p.f.u. per cell in one of these suspensions as compared with the average yields of 19 and 24 p.f.u. per cell in the other two cultures might have been the

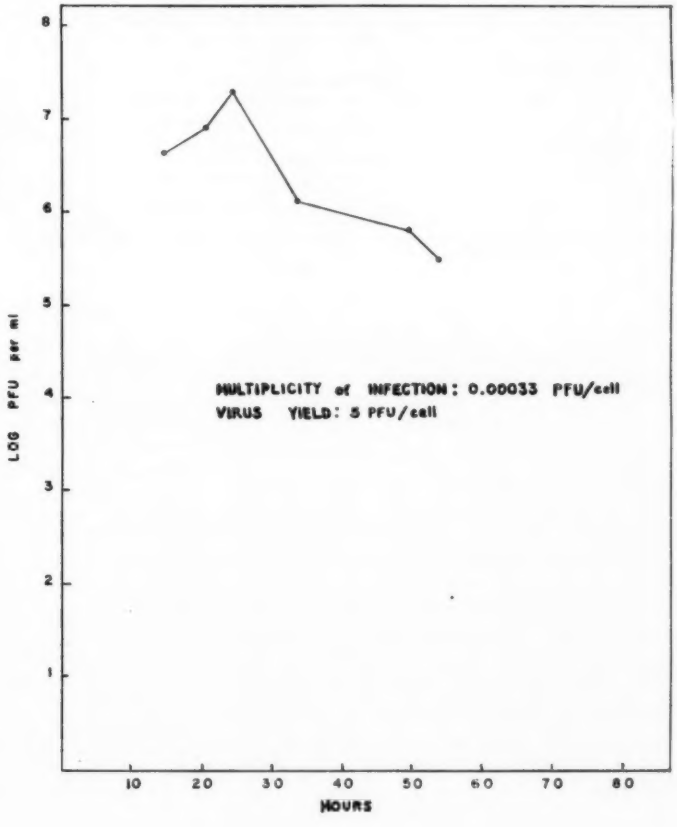

Fig. 3—Growth of foot-and-mouth disease virus A119 in a 10-liter suspension of trypsin-dispersed bovine kidney cells.

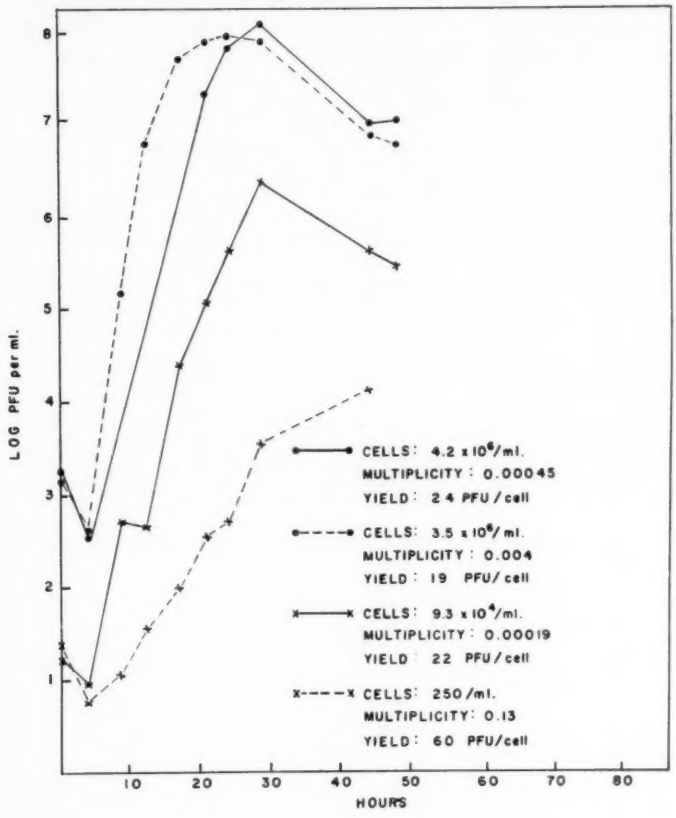

Fig. 4—Growth of foot-and-mouth disease virus A119 in suspensions of trypsin-dispersed bovine kidney cells grown first on glass.

result of a more favorable virus-cell ratio at the time of inoculation.

SUMMARY

Suspended cultures were prepared from bovine kidney tissue in which minced tissue, trypsin-dispersed cells, and trypsin-dispersed cells from cultures first grown on glass were maintained in a viable state. Such suspensions were infected with foot-and-mouth disease virus, type A, strain 119, and high titers were attained. Suspensions of minced tissue, trypsin-dispersed cells, and trypsin-dispersed cells first grown on glass produced maximum titers of $10^{6.2}$, $10^{7.2}$, and 10^{8} plaque-forming units (p.f.u.) per milliliter, respectively, in 40-ml. cultures.

A yield of $10^{7.2}$ p.f.u. per milliliter from a 10-liter suspended culture of trypsin-dispersed cells indicated the feasibility of the production of large volumes of high-titered virus by the suspension method.

The saving in materials, time, and labor by the suspension method over that of the monolayer method is discussed.

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Spontaneous Tumors in Primates—A Report of Two Cases with Notes on the Apparent Low Incidence of Neoplasms in Subhuman Primates

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AVAILABLE EVIDENCE indicates that susceptibility to neoplasms may be low in primates other than man; therefore, spontaneous neoplasms in these species are of considerable current interest. Two tumors which apparently have not been previously observed in subhuman primates are reported here, and the apparent resistance of monkeys and apes to neoplasms is discussed.

CASE 1

One of the tumors was found in a female cynomolgus monkey *(Macaca philippinensis)* procured from the Philippine Islands and estimated to be between 2 and 3 years old. No prior illness had been observed and the monkey had been killed for use in the production of poliomyelitis vaccine.

Necropsy Findings.—At necropsy, a tumorous mass was found which appeared to involve the left ovary, fallopian tube, and anterior aspect of the uterus (fig. 1). It measured approximately 3 by 6 cm. and was partially bilobed. One half was mottled from hemorrhage; the other half was firm and white, and the cut surface glistened. No delineation between uterine and ovarian mass was demonstrated grossly. The right ovary and fallopian tube, the posterior half of the uterus, and the cervix appeared to be normal.

Microscopic Findings.—Low-power microscopic examination revealed areas in which tumor cells had begun to invade and efface the stroma or glandular elements (or both) of the myometrium, endometrium, and ovary. There were numerous focal hemorrhages and marked deposition of hemosiderin. Sections from the uterine area (fig. 2) showed masses of tumor cells separated by fibrous connective tissue and smooth muscle within which endometrial glands occasionally were found. By contrast, sections from the ovarian area contained tumor cells, ovarian stromal components and, not infrequently, immature follicles.

High-power magnification of uterine and ovarian areas revealed tumorous masses of anaplastic epithelial cells which occurred as continuous sheets, as isolated masses of two or more cells lying in the stroma, or occasionally as poorly formed glands without prominent lumens. The individual epithelial tumor cells were characteristically large and undifferentiated, with clear cytoplasm and large vesicular nuclei with hyperchromatic nucleoli (fig. 3). Many were undergoing atypical mitosis.

The tumor was classified as an undifferentiated carcinoma involving both uterus and ovary. Its origin was not determined.

CASE 2

The other tumor was found in a male gibbon (Hylobates sp.) of unknown age which had been procured from India and housed in a zoo in Miami, Fla.*

The attending veterinarian reported that for approximately one week prior to death it had been anorectic, lethargic, dehydrated, markedly anemic, and had intermittent fever. An erythrocyte count revealed less than a million cells per cubic millimeter of blood. No other laboratory work was performed. Despite supportive treatment the condition rapidly became worse and the gibbon died about one week after illness was first noticed.

Necropsy Findings.—The tissues other than the viscera appeared blanched, as if anemic. The gastrointestinal tract contained a large amount of mucus but no food. The mucosa of the intestine and, to a lesser degree, the stomach, was markedly congested and showed several ecchymotic hemorrhages. The intestinal wall appeared thickened in several areas. The lymph nodes and spleen were moderately enlarged,

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The authors appreciate the assistance of Dr. Karl Mayer, Lilah Estill, and Mary Ellen Weidlich in the preparation of this report.

* Tissues submitted by Dr. N. E. Bowen, Miami, Fla.

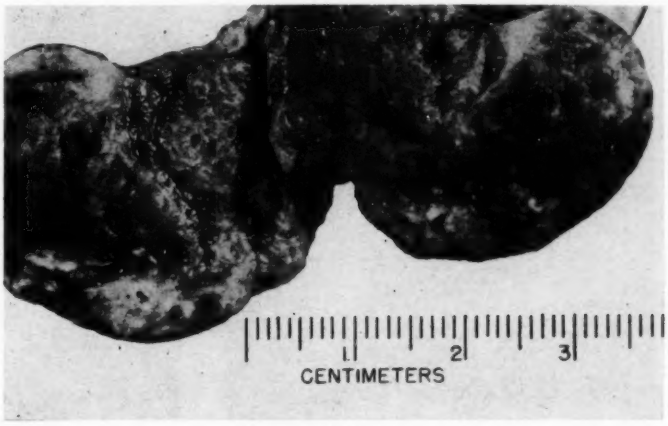

Fig. 1—Tumor involving uterus and ovary of a cynomolgus monkey. Notice darkened areas of hemorrhage in the otherwise firm, glistening mass.

and both the liver and kidneys were studded with grayish white foci which were firm and glistened when sectioned. The heart crypts contained several large, grayish blood clots. The lungs were essentially normal grossly. Representative portions of these organs were referred to this laboratory for histologic study.

Microscopic Findings.—The foci in the liver and kidney were found microscopically to be composed of anaplastic lymphoid cells with prominent, hyperchromatic nuclei and scanty cytoplasm. There were many mitotic cells. The architecture of the lymph nodes was partially effaced by a uniform overgrowth of tumor cells. The splenic structures also were altered, although not completed effaced, by diffuse distribution of neoplastic cells in both the red and white pulp. Occasionally a blood vessel was found in which the wall appeared to have been invaded, and small masses of tumor cells were seen lying free in the lumen or in association with erythrocytes (fig. 4). Sections of the liver revealed neoplastic cells in the portal spaces, the sinusoids of the lobules, and the subcapsular areas (fig. 5, 6). An occasional blood vessel appeared to be invaded, in which case tumor cells were usually found in the lumen. Many of the mesenteric blood vessels also contained free masses of neoplastic

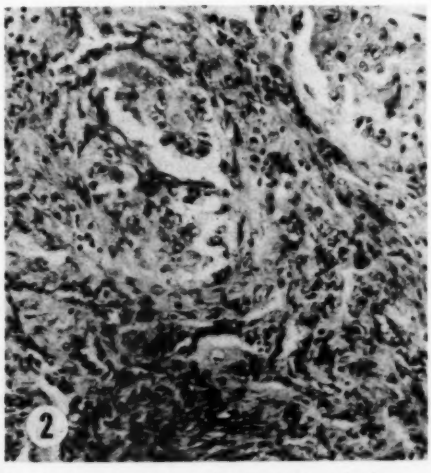

Fig. 2—Low-power view of tumor shown in figure 1. H & E stain; x 100.

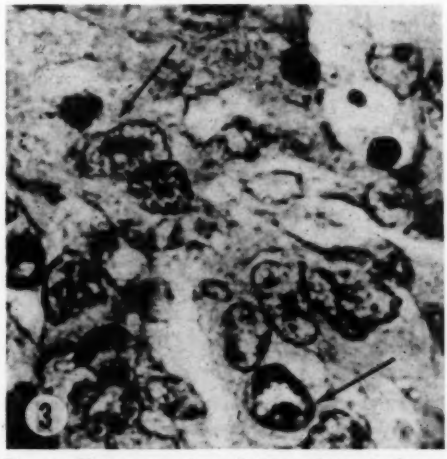

Fig. 3—High-power view of tumor shown in figures 1 and 2. Notice anaplastic features of individual epithelial cells (arrows). H & E stain; x 465.

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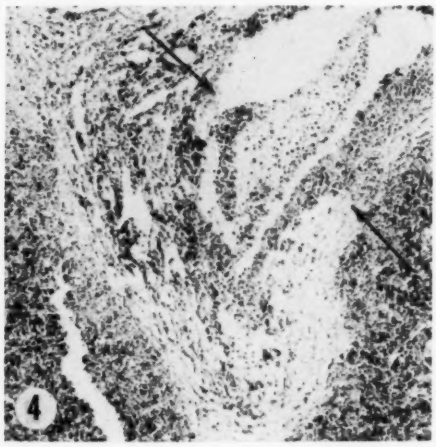

Fig. 4—Section of spleen from gibbon. Notice tumor cells both in blood vessel wall and lumen (arrows). H & E stain; x 65.

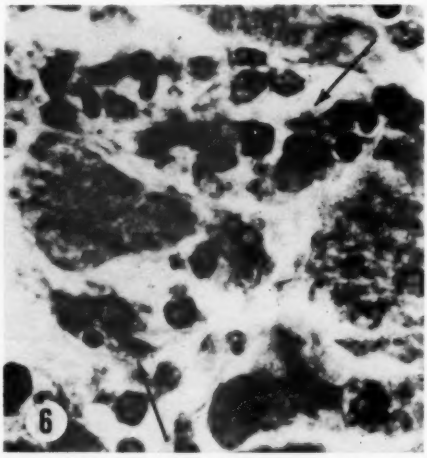

Fig. 6—Higher magnification of liver section shown in figure 5. Notice neoplastic cells in sinusoids (arrows). H & E stain; x 465.

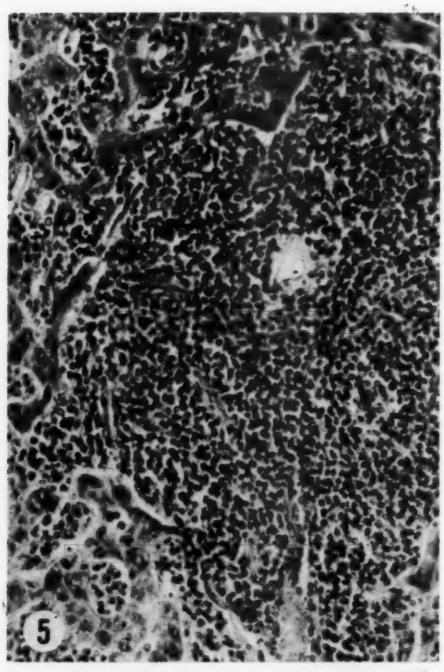

Fig. 5—Section of liver from gibbon. Notice massive invasion of sinusoids and triads with anaplastic lymphoid cells. H & E stain; x 100.

lymphoid cells (fig. 7). In the kidneys, the interstitial areas were heavily infiltrated with neoplastic cells which had effaced and replaced many of the tubules, particularly in the cortex (fig. 8, 9). Sections of intestine showed tumor cells to be invading the lamina propria and infiltrating from this site into the mucosal lining cells. There was no apparent invasion of the musculature. The lungs were essentially normal, except that close study occasionally revealed neoplastic cells in the capillaries and larger blood spaces. In the heart (fig. 10, 11), several large masses of neoplastic cells were seen lying free or in association with other formed elements in the vascular spaces. However, none were seen invading the myocardium.

The tumor was classified as a malignant lymphoma. In view of the large number of tumor cells demonstrated in association with other formed elements in the vascular spaces of the heart and other organs, there appears to be considerable evidence that a concomitant leukemic state existed. Moore's[6] description and illustrations of chronic lymphoid leukemia in man, which he lists as a variant of malignant lyphoma, compared rather closely with the observations we have made of the tumor in the gibbon.

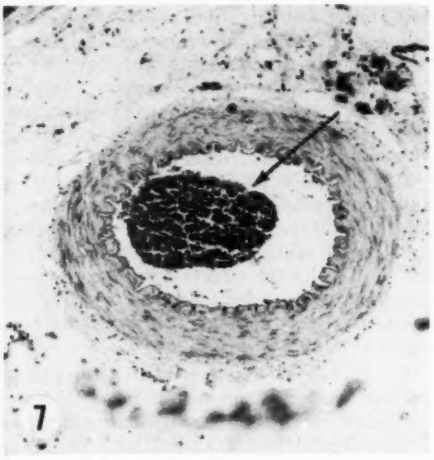

Fig. 7—Mesenteric artery from gibbon showing unattached mass of neoplastic lymphoid cells in the lumen (arrow). H & E stain; x 65.

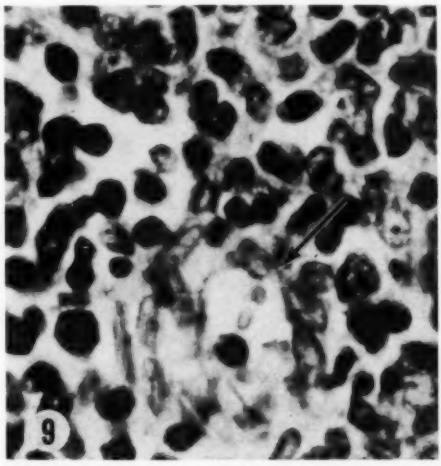

Fig. 9—Higher magnification of section shown in figure 8. Notice effacement of tubule (arrow) with neoplastic cells. H & E stain; x 465.

Discussion

Few spontaneous neoplasms have been reported from subhuman primates.[1-4] Furthermore, at the time of this writing, no spontaneous tumors have been reported in the many thousands of monkeys used by various groups during the past several years in the development, production, and testing of poliomyelitis vaccine. Although their actual status with regard to tumors is uncertain, it is reasonable to assume that any great incidence of tumors in these monkeys would probably have been reported. In our own series of more than 100,000 monkey necropsies, we have found only one tumor, reported here in the cynomolgus monkey, giving further support to the view that the over-all incidence of spontaneous neoplasms in subhuman primates is extremely low.

Several factors should be considered before any conclusions are drawn regarding the apparent resistance of subhuman primates to neoplasms. The age at death is among the most important. Only a limited number of subhuman primates necropsied, principally those in zoos, have reached an age comparable to the age at which the highest incidence of cancer is encountered in man. A majority of those used experimentally and for vaccine production have

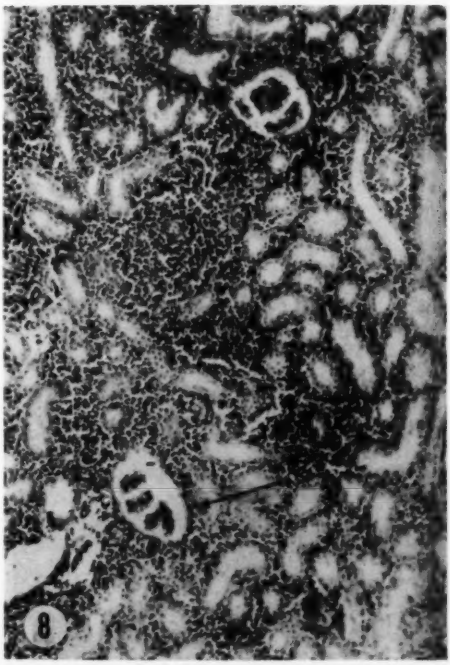

Fig. 8—Section from kidney of gibbon. Notice marked interstitial invasion with neoplastic cells. Arrows indicate glomeruli. H & E stain; x 65.

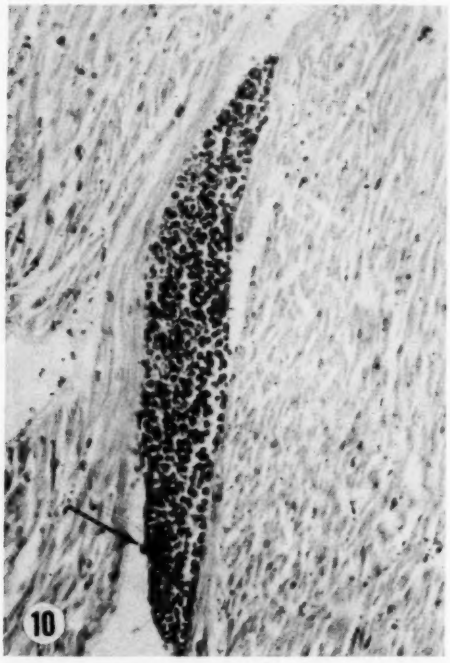

Fig. 10—Section from heart of gibbon. Notice unattached mass of tumor cells in vascular space (arrow). H & E stain; x 100.

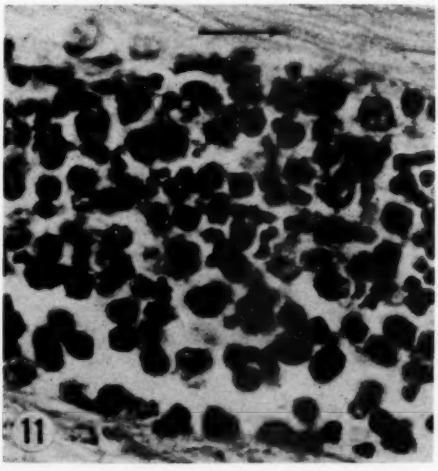

Fig. 11—Higher magnification of tumor mass shown in figure 10. Notice anaplastic features of tumor cells and absence of myocardial invasion (arrow). H & E stain; x 455

been young animals, and many others have died early in life of diseases resulting from environmental factors in captivity. It is also quite probable that some small tumors have been overlooked at necropsy. It must, therefore, be concluded that the known incidence of tumors in this group of animals would probably be increased somewhat if conditions were optimal with respect to age and the exactness of necropsy procedures. However, it seems unlikely that even optimal conditions for tumor development and diagnosis would very markedly modify the apparently low incidence indicated by currently available data.

The fact that experimental attempts to produce cancer in monkeys have, for the most part, been unsuccessful lends support to this view. Pfeiffer and Allen [7] failed to produce neoplasms in monkeys cutaneously exposed for more than ten years to hydrocarbons and estrogens, which are known to be carcinogenic in other species. Other investigators [5, 8] have had only slightly better results using motor lubricating oil and a petroleum derivative. Kent and Pickering [3] recently reported that ionizing radiation might be a more consistently successful method for producing tumors experimentally in monkeys. In general, however, it has not been shown that neoplasms can be consistently produced in monkeys by any of the methods that are currently successful with other species. The difficulties which various investigators have encountered in producing neoplasms in these animals would appear to be related to the low recorded incidence of spontaneous neoplasms.

SUMMARY

An undifferentiated carcinoma involving the uterus and ovary of a cynomolgus monkey, and a malignant lymphoma in a gibbon are described. The former was the only neoplasm encountered in a series of more than 100,000 necropsies of monkeys. The latter was found in material submitted from a zoo for routine histologic study. This apparently is the first time that either of these neoplasms has been observed in these species. Currently available evidence from necropsies, as well as from various attempts to induce tumors experimentally, suggests that the subhuman primates have a low susceptibility to tumor formation.

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Scientific Notes

Chlorpromazine Premedication with Pentobarbital Anesthesia in a Mouse

William C. DOLOWY, M.S., D.V.M.; Paola MOMBELLONI, M.D.; A. L. HESSE, B.S.

Chicago, Illinois

PENTOBARBITAL SODIUM given alone results in unsatisfactory anesthesia for major surgery in mice because of the great variation in response of individuals. Improved results reported with chlorpromazine hydrochloride as a preanesthetic in dogs,[1] cats,[2] rabbits,[3] and rats [4] led to investigation of its effects in mice.

PROCEDURES

Duration of surgical anesthesia in mice was observed using the following signs as criteria: absence of righting reflex, appearance of deep sleep, absence of toe-pinch response, and absence of tensing of abdominal muscles upon repeated pin-pricking of abdominal skin from sternum to pubis.

Sixty female Harlan albino mice, allotted to six groups, were anesthetized with pentobarbital sodium given intraperitoneally in doses of 65 to 120 mg. per kilogram of body weight (table 1). The pentobarbital was freshly diluted in pyrogen-free water.

Another 60 female Harlan albino mice were allotted to five groups and preanesthetized with chlorpromazine hydrochloride given intramuscularly in doses of 25 to 60 mg./kg. This was followed by intraperitoneal injection of pentobarbital sodium in doses of 40 to 60 mg./kg. (table 2).

All mice were observed for two weeks following recovery from anesthesia. Livers from mice given 90 mg./kg. of pentobarbital alone and from mice given 50 mg./kg. of chlorpromazine plus 50 mg./kg. of pentobarbital were examined grossly and histologically with both the hematoxylin and the Sudan IV stains.

RESULTS AND CONCLUSIONS

Duration of surgical anesthesia and survival rates of mice in the two experiments are recorded (tables 1, 2). Chlorpromazine hydrochloride given intramuscularly at the rate of 50 mg./kg. and followed by pentobarbital sodium given intraperitoneally resulted in a longer average duration of surgical anesthesia (22 min.) than any other dose of a single drug or combination of drugs with equal (87%) or higher survival rates and with 80 per cent successful surgical anesthesia.

The gross and histologic appearance of the livers of the 10 mice given 90 mg./kg. of pentobarbital sodium alone was normal. The gross appearance of the livers of 10 mice given 50 mg./kg. of chlorpromazine and 50 mg./kg. of pentobarbital was normal, but histologic examination revealed an increased granularity of cytoplasm

From the Animal Hospital and the Department of Pathology, College of Medicine, Chicago Professional Colleges, University of Illinois, Chicago.

TABLE 1—Duration of Surgical Anesthesia and Survival Rates in Female Albino Mice (Harlan) After Intraperitoneal Injection of 0.5 Per Cent Pentobarbital Sodium

Group *	Pentobarbital Na (mg./kg.)	Animals in surgical anesthesia (%)	Survivors (%)		Av. duration and range of surgical anesthesia in survivors (min.)
			1 day	1 week	
1	65	20	90	90	3.6 (0–8)
2	80	30	100	80	3.6 (0–19)
3	90	80	80	80	6.0 (0–20)
4	100	100	70	70	111.0 (109–115)
5	110	100	40	40	124.0 (84–168)
6	120	100	20	20	127.0 (123–132)

* Ten animals were used per group.

TABLE 2—Duration of Surgical Anesthesia and Survival Rates in Female Albino Mice (Harlan) After Injection of 0.5 Per Cent Chlorpromazine Hydrochloride Intramuscularly, Followed by 0.5 Per Cent Pentobarbital Sodium Intraperitoneally 30 Minutes Later

Group	No. of animals	Chlorpromazine hydrochloride (mg./kg.)	Pento-barbital Na (mg./kg.)	Animals in surgical anesthesia (%)	Survivors (%)		Av. duration and range of surgical anesthesia in survivors (min.)
					1 day	1 week	
7	5	25	40	40	80	80	82 (0–95)
8	5	50	40	20	80	80	95 (0–95)
9	30	50	50	80	87	87	22 (0–51)
10	10	60	50	50	100	80	17 (0–56)
11	10	60	60	100	80	60	78 (67–99)

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and a less intense uptake of stains in the hematoxylin and eosin preparations.

The stimulating effect of chlorpromazine on the respiratory rate [5] is probably responsible for the success of this combination.

Additional research with preanesthestics is indicated to improve the safety and duration of general anesthesia in the mouse.

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Meetings • • •

• • • University of Nebraska Conference on Swine Repopulation. University of Nebraska, College of Agriculture, Agricultural Experiment Station, Lincoln, Feb. 3–5, 1960. Dr. E. Crosby Howe, Department of Animal Pathology and Hygiene, University of Nebraska, Lincoln 3, Nebr.

• • • American College of Radiology, New Orleans, La., Feb. 3–6, 1960. Mr. William C. Stronach, 20 N. Wacker Dr., Chicago 6, Ill., executive director.

• • • American Association of Pathologists and Bacteriologists. Memphis, Tenn., April 28–30, 1960. Dr. Russell L. Holman, Department of Pathology, Louisiana State University School of Medicine, New Orleans, La., secretary.

• • • Society of American Bacteriologists. Philadelphia, Pa., May 1–5, 1960. Dr. E. M. Foster, 311 Bacteriology, University of Wisconsin, Madison 6, secretary.

• • • American Federation for Clinical Research. Atlantic City, N.J., May 2, 1960. Mr. James E. Bryan, 250 W. 57th St., New York 19, N.Y., executive secretary.

• • • Rare Earths in Biochemical and Medical Research Conference. Iowa State University, Ames, May 11–13, 1960. Dr. J. G. Graca, College of Veterinary Medicine, Iowa State University, Ames, program chairman.

• • • International Association of Veterinary Food Hygienists, Second Symposium. Basel, Switzerland, May 15–21, 1960. Dr. A. Clarenburg, 1 Sterrenbos, Utrecht, Netherlands, president.

• • • International Congress of Clinical Pathology. Madrid, Spain, June 13–17, 1960. Dr. J. Aparicio Garrido, Sandoval 7, Madrid, Spain, secretary-general.

• • • First International Congress of Endocrinology. Technical University of Denmark, Copenhagen, July 18–23, 1960. Dr. Svend G. Johnsen, Statens Seruminstitut, Copenhagen 5, Denmark, congress secretariat.

Fifth International Congress on Nutrition

The Fifth International Congress on Nutrition will be held at the Sheraton Park and Shoreham Hotels in Washington, D.C., Sept. 1–7, 1960.

An all-day symposium on "World Food Needs and Food Resources" will be the main feature of the scientific program; there will also be seven half-day sessions featuring three-hour panel discussions. Along with the panel discussions there will be special programs of ten-minute papers reporting unpublished original research. The subjects of these special programs will be:

1) Preservation of Foods by Ionizing Radiation
2) Metabolism of Fission Products
3) Use of Radioisotopes in Nutrition Research
4) Nutritional Physiology of Ruminants
5) New Aspects of Carbohydrate Metabolism
6) Micro-nutrients in Plant and Animal Nutrition
7) Functional Role of Vitamins
8) Human Nutritional Requirements and Allowances
9) Animal Nutrition at the Cellular Level

Preference will be given to reports related to these nine topics; deadline for abstracts of papers is April 1, 1960.

In addition to the formal program, there will be exhibits of laboratory instruments, books, foods, and other products. Visits to scientific laboratories in the Washington area are also planned.

For further information concerning the program, registration, and submission of papers, contact the Secretariat, 9650 Wisconsin Avenue, Washington 14, D.C.

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International Congress on Animal Reproduction Postponed Until 1961

The Fourth International Congress on Animal Reproduction was to be held in June, 1960, at Amsterdam, Netherlands. After consultation with the International Committee for Animal Reproduction and Artificial Insemination, it was decided that the congress would be postponed for one year so that members would be able to attend the Eighth International Congress of Animal Husbandry, which will be held in Hamburg on June 13, 1961. The new date and location will be June 5–9, 1961, The Hague, Netherlands.

Those interested may apply to: Secretariat of the Fourth International Congress on Animal Reproduction, 14, Burgemeester de Monchyplein, The Hague, Netherlands.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

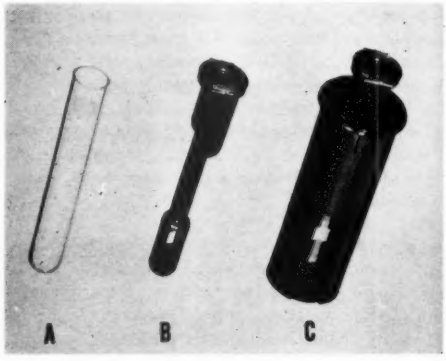
Ultraviolet Analysis Cabinet

An ultraviolet analysis cabinet for chromatography (Chromato-Vue, manufactured by Ultra-Violet Products, Inc.) contains sources of near ultraviolet, far ultraviolet, and white ultraviolet light.

This instrument is designed to simplify the examination of fluorescent chromatograms in brightly illuminated laboratories. Long wave (3,660 Å) and short wave (2,537 Å) lamps can be used independently or in combination. The lamps fit into cradles in the cabinet and can be adjusted to any of four vertical positions. The white light is used for illumination when marking chromatograms. The flexible foam eyepiece is mounted over an ultraviolet-absorbing glass; a wide front opening permits easy insertion of chromatograms into the cabinet. This opening is covered with a double-thickness black fabric curtain to shut out extraneous light.

Spectrophotometer Micro Accessories

Micro accessories for use with the Coleman Junior Spectrophotmeter are being manufactured by Coleman Instruments, Inc.

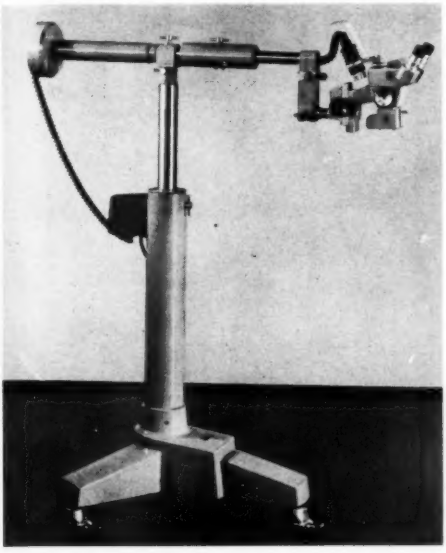


These accessories are useful in measurements where the sample size is small and concentration is low. Precise spectrophotometric measurements through a full 1.0 cm. path can be made with a solution volume of only 0.7 ml. The precision-bore cuvettes (A, "Perfect Rounds") have an internal diameter of 10 mm. They are positioned in the monochromatic beam by a Coleman Cuvette Adapter (C) and may be used with or without the Coleman Micro-Space Adapter (B). When used alone, they require a minimum sample volume of 1.8 ml. The micro-space adapter reduces this requirement to 0.7 ml.

3-D Surgical Microscope

A new surgical microscope with 3-dimensional magnification for surgical procedures and clinical examination is available from the American Optical Company.

This microscope will be useful for observation of minute body surfaces or detail deep inside small apertures. Magnification can be changed easily with a dial. When the microscope is in focus,



there is ample space between the specimen area and the objective (8 inches) for hands and surgical instruments. An adjustable vertical pillar and horizontal arm permit a wide range of adjustment.

Automatic Microtome Sharpener

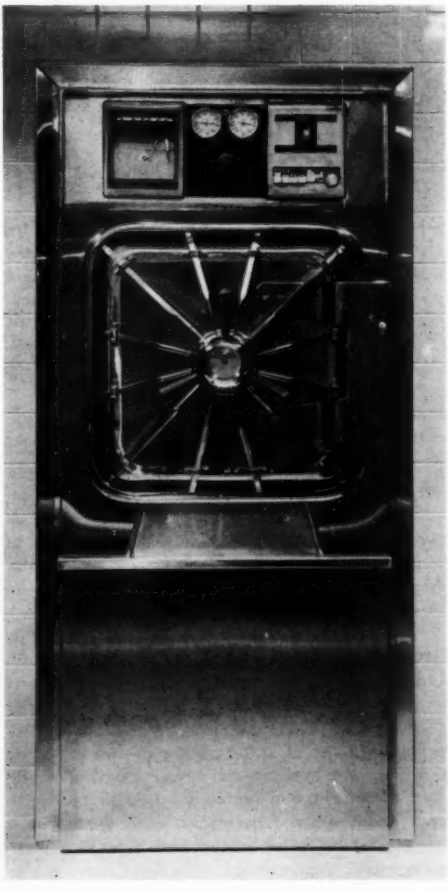
The Spencer Automatic Knife Sharpener, manufactured by the American Optical Company, sharpens microtomes up to 185 mm. in length.

The instrument has a high-frequency vibrating glass honing plate, a gravity-fed water lubrication system, and an electrical timing device. A transparent plexiglass cover provides a dust and grit-free process. The abrasive powder which is applied to the interchangeable ground glass honing plate comes in two grades, coarse for sharpening, and fine for polishing. The device operates on any standard 110v outlet.

Automatic Sterilizer

A completely automatic sterilizer (Castle Orthomatic, manufactured by Wilmot Castle Co.) reduces cycle time by half.

An electromatic jet heating system, using full steam line pressure, speeds the heating phase. Temperature can be varied from 220 to 270 F. The cooling system cools a full liquid load in an average of twenty minutes. Fresh filtered air, passed through a sterile bacteria-retentive filter, affords rapid drying. The drying timer is calibrated to the minute.

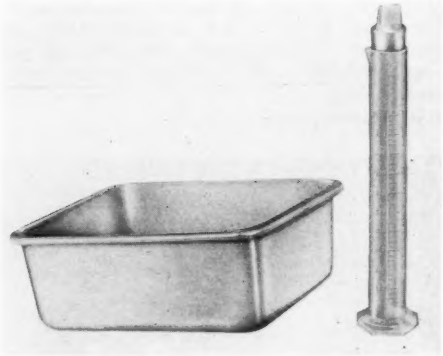


The sterilizing cycle is started by pushing one of four keys (liquids, dry goods, manual, steam off); subsequent phases follow automatically. An illuminated color panel indicates the cycle phase, and a buzzer sounds when the cycle is complete.

The sterilizer is available in either a cabinet or recessed mounting, with a cylindrical, straight-line, or rectangular body.

Polypropylene Laboratory Tools

A series of graduated mixing cylinders, as well as a sterilizing pan, are being manufactured in unbreakable polypropylene by the Nalge Company.



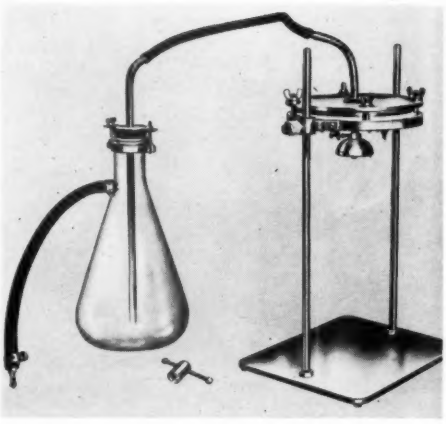
Polyethylene stoppers have been designed for use with the mixing cylinders. Volumes are easily read, since water solutions in polypropylene have almost no meniscus. Accuracy is within 1 per cent. These graduated cylinders are available in six sizes; all dimensions are in accordance with federal specifications for glass.

The sterilizing pan, 12½ x 10 x 5¼ inches, is designed for sterilizing or drying glassware, instruments, and other materials at elevated temperatures.

Paper Chromatography Aid→

An apparatus for fast and easy handling of large numbers of paper strips for one-dimensional chromatography is available from the California Laboratory Equipment Co.

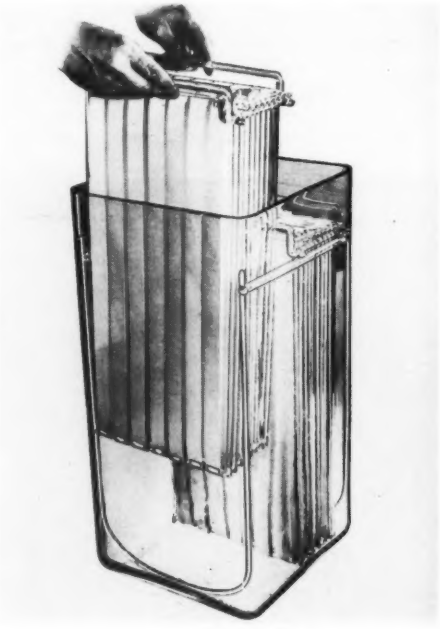
Paper strips are stretched on specially designed glass frames; the all-glass apparatus eliminates fumbling with wet, dangling paper strips and prevents papers from touching or rubbing together while being developed, washed, sprayed, and dried. A large number of strips can be processed at one time in a small space and under identical conditions of solvent and humidity. Anchor and anti-siphon rods are not necessary. The apparatus is offered for both descending and ascending chromatographic separations.



Laboratory Filters

Laboratory filters, manufactured by Ertel Engineering Corp., are available in stainless steel and nickel or silver-plated bronze.

Double filtration is automatically performed. Two pyrogen and bacterial retentive filter sheets are located one above the other and are separated by a collecting ring. Liquid passes through the upper filter sheet and accumulates in the collector ring until pressure forces it through the lower filter sheet. A built-in fiber eliminator prevents the passage of fiber.

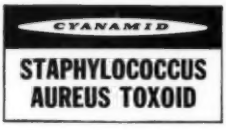


Staphylococcus Aureus Toxoid
Slanetz Strain No. 7

This product, developed by Dr. L. W. Slanetz and associates at the University of New Hampshire for the control of staphylococcal mastitis*, now is available to the veterinary profession.

For further information, please drop a card to the address below or ask your local Cyanamid representative for details.

Veterinary Professional Service Department
American Cyanamid Company
New York 20, N. Y.



*Journal A.V.M.A. 134:155-161 (Febr. 15) 1959

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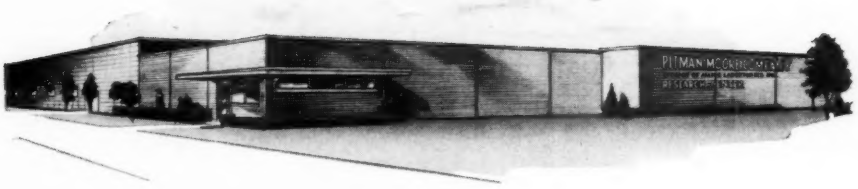


new research facilities for

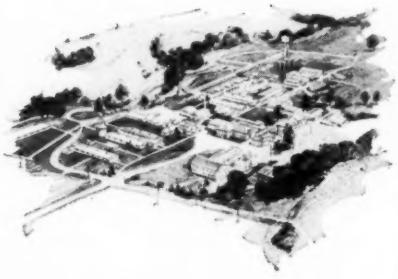
PITMAN-MOORE COMPANY

Division of Allied Laboratories, Inc.

A $1,500,000 building, completed early in 1959, enables Pitman-Moore Company to concentrate in one location all types of research contributing to the development of human and veterinary medical preparations. This new unit adds 41,000 square feet of space for laboratories, offices, and technical equipment in the company's Research Center near Zionsville, Indiana.

The Indianapolis laboratories, where pharmaceuticals are produced.

The Zionsville laboratories, where biologicals are produced.



Within this organization originated the policy: Sales to Graduate Veterinarians ONLY

PITMAN-MOORE COMPANY
DIVISION OF
ALLIED LABORATORIES, INC.
INDIANAPOLIS 6, INDIANA